Local somatic hypermutation and class switch recombination in allergic rhinitis

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LOCAL SOMATIC HYPERMUTATION AND CLASS SWITCH RECOMBINATION IN ALLERGIC RHINITIS

by

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Abstract

This thesis documents research that investigated whether class switch recombination and somatic hypermutation occur locally in the nasal mucosa of allergic rhinitis patients. Analysis of V_H region sequences from the nasal mucosa demonstrated families of related IgE^+ B cell clones, exhibiting identical signature region sequences and both shared and differing V_H region mutations. V_H region sequences from IgE^+ B cells isolated from adjacent pieces of allergic nasal mucosa demonstrated that members of an IgE^+ B cell clonal family present in, for example, one half of a nasal biopsy, could not be detected in the other half. V_H region sequences were also detected from IgA^+ B cells, related to an IgE^+ B cell clonal family.

These observations, strengthened by the presence of mRNA encoding AID, support the concept of local class switch recombination, somatic hypermutation and clonal expansion in the allergic nasal mucosa. Furthermore, the V_H5 class of genes was over-represented in sequences isolated from nasal mucosa IgE^+ B cells. In combination with unusual non-intrinsic hotspots of mutation in the V_H5 sequences, this was suggestive of local selection of some IgE^+ B cells by a B cell superantigen in the allergic nasal mucosa.

It is therefore suggested that in allergic rhinitis, the nasal mucosa is an organised microenvironment in which local class switch recombination, somatic hypermutation, clonal expansion and antigen selection occur, optimising the efficacy of the local allergic response.

This thesis is dedicated to the memory of my father Peter Coker, who could not have given me more love and support.

(1947 – 2003)

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Abbreviations

A	Adenine
AID	Activation-induced cytidine deaminase
BALT	Bronchial associated lymphoid tissue
C	Cytosine
C_H	Constant heavy chain region
CDR	Complementarity determining region
CSR	Class switch recombination
(c)DNA	(Copy) deoxyribonucleic acid
D region	Diversity region
Fab	Includes the antigen binding portion of an antibody
Fc	Includes the receptor binding portion of an antibody
FcεRI	IgE high affinity receptor
FcεRII	IgE low affinity receptor (CD23)
FcR	Fc receptor
FDC	Follicular dendritic cell
FWR	Framework region
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H	Antibody heavy chain
HDM	House dust mite
HLA-DR	A form of the human major histocompatibility, class II complex
I	Heavy chain gene promotor
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
J_H	Heavy chain joining region
L	Antibody light chain
MHC	Major histocompatibility complex
(m)RNA	(Messenger) ribonucleic acid
N region	Non-templated nucleotide insertion
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
R	Purine (A or G)

RAG	Recombination-activating gene
RSS	Recombination signal sequence
RAST	Radioallergosorbency test
R/S	Ratio of replacement to silent nucleotide mutations
RT	Reverse transcription
S	Switch region
SCT	Switch circle transcript
SHM	Somatic hypermutation
T	Thymine
U	Uracil
UNG	Uracil-DNA glycosylase
UV	Ultraviolet light
V_H region	Heavy chain variable region
V_L region	Light chain variable region
W	A or T
Y	Pyrimidine (C or T)

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Chapter 1

Introduction

1.1 An introduction to allergy.

Allergic disease has become of increasing concern to developed countries, where the incidence of allergy can be so great that a quarter of children under fourteen suffer from asthma [Beasley, 1998]. While strictly an allergic reaction can result from several different mechanisms, the term 'allergy' usually refers to type I hypersensitivity, mediated by antibodies of the immunoglobulin E (IgE) isotype. It is this definition of allergy that will be used in this thesis. Allergy may manifest as a variety of diseases from which a patient may suffer with single or multiple forms, allergic rhinitis, allergic asthma and allergic dermatitis being the most common. In all allergic disease, IgE-mediated hypersensitivity is a consequence of the interaction of allergen with IgE. Allergens originate from a huge variety of different origins and cannot be identified by a single common property [Gould *et al.*, 2003]. A patient may be allergic to single or multiple allergens.

Allergy is a multifactorial disease, contributed to by both genetic and environmental factors. There are multiple genetic markers for allergy, including particular HLA-DR alleles, polymorphisms of FcεRI (the high affinity receptor for IgE) and polymorphisms in interleukin-4 (IL-4). The cumulative effects of the different genetic markers for allergy result in a less than 15% chance of a child with non-allergic parents developing atopy (a clinical predisposition to allergy), while if one parent is allergic there is nearly a 30% chance, and if both parents are allergic the likelihood is increased to more than 50% [Roitt *et al.*, 1996].

Even if a subject has inherited a predisposition towards atopy, environmental factors may be important in determining if they develop clinical symptoms, *i.e.* if they are allergic. Environmental pollutants are thought to exacerbate allergic disease and diesel exhaust particles, for example, have been shown to increase production of IgE detected in nasal lavage fluid from human subjects [Diaz-Sanchez *et al.*, 1994].

Exposure to both bacteria and allergens can also influence the development of atopy. A bias towards T_H2 cells and away from the T_H1 cell type necessary in the immune response against bacteria, is characteristic of allergy. The essential role of T_H2 cells in allergy is described in greater detail in section 1.3, but it has been suggested that while it is usual for the foetus to exhibit a T_H2 bias, after birth, exposure to bacterial antigens

results in the development of a T_H1 biased repertoire of cells. In Westernised countries it has therefore been hypothesized that the decreased exposure of neonates to bacteria results in the maintenance of the T_H2 bias, particularly in children with atopic parents [reviewed by Kay, 2001]. Exposure to high levels of allergen, such as that from house dust mite, is also far more common in Westernised society [reviewed by Kay, 2001].

1.2 Allergic rhinitis.

Allergic rhinitis is the manifestation of allergic disease in the nose. Patients suffer from sneezing, itching, rhinorrhea and nasal obstruction [reviewed by Kay, 2001b]. Rhinitis is not always allergic in origin and so, as with other allergic disease, is determined on the basis of both a positive skin-prick test and also elevated allergen-specific serum IgE (determined by radioallergosorbency test (RAST)) [Ying *et al.*, 2001], combined with a clinical history of rhinitis symptoms, for diagnosis. Only such conventional allergic rhinitis patients were included in the research presented in this thesis. However, some patients, while displaying classic symptoms of allergic rhinitis, exhibit both negative skin-prick test and RAST results. It has been suggested that these patients, known as non-atopic rhinitics (or intrinsic rhinitics), suffer either from an allergy towards an unidentified allergen, or that the allergic response is confined solely to the nasal mucosa [discussed by Kay, 2001b).

Allergic rhinitis can occur as both a seasonal and / or perennial disease. In the temperate northern hemisphere, seasonal rhinitis is usually a consequence of either tree (in spring), or grass (in summer) pollen allergy. In contrast, perennial rhinitis patients are sensitive to allergens such as house dust mite or animal dander, present throughout the year.

In the nose there are three nasal turbinates in each nostril, the inferior, middle and superior. These are situated on the opposite wall of the nose to the septum (*Fig.* 1.2.1). The nasal turbinates contain a high concentration of immune cells and as a consequence are often swollen, especially in perennial allergic rhinitis patients [Mygind *et al.*, 1996]. Research conducted into allergic rhinitis often relies on analysis of the nasal mucosa of the inferior turbinate (being the nearest to the entry point of aeroallergens to the nose). Samples of the inferior turbinate are most commonly obtained by nasal biopsy, although

when allergic rhinitis results in very severe nasal obstruction, surgical removal of the whole inferior turbinate is an extreme solution.

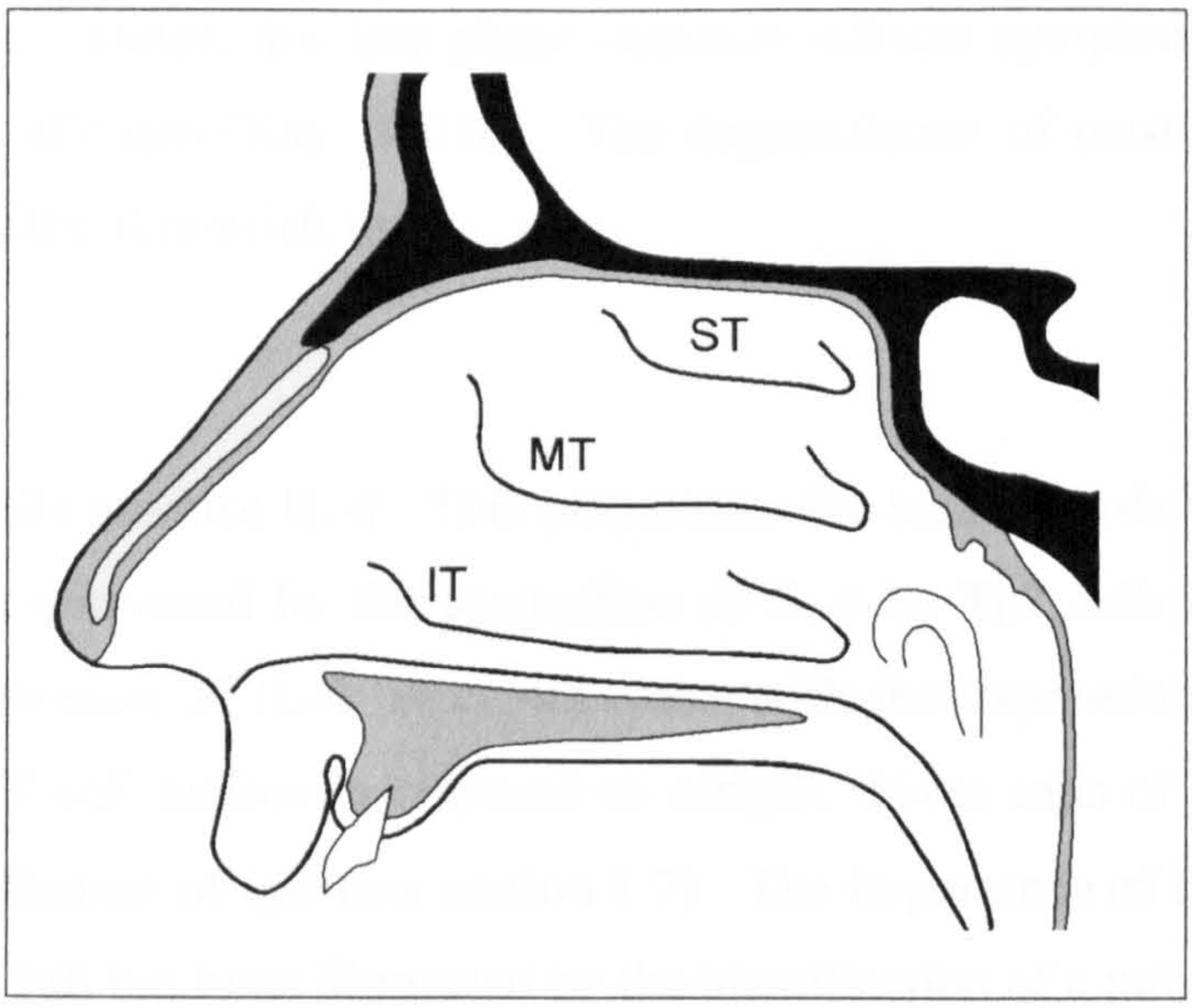


Fig. 1.2.1; The position of the inferior, middle and superior turbinates in the nasal cavity [adapted from Mygind et al., 1996]. The site of the inferior (IT), middle (MT) and superior (ST) turbinates on the opposite side of the nasal cavity to the septum are detailed.

1.3 The mechanisms of IgE-mediated hypersensitivity.

IgE is produced by plasma B cells and is known to occur locally in the nasal mucosa of allergic rhinitis patients [Smurthwaite *et al.*, 2001]. Allergen–specific IgE is produced locally throughout the year, even in seasonal allergic rhinitis patients [Smurthwaite *et al.*, 2001]. IgE has two receptors to which it can bind with either high (FcεRI), or low affinity (FcεRII, also known as CD23). CD23 is present both as a surface-bound and soluble molecule and is involved in complex feedback mechanisms controlling IgE synthesis [reviewed by Gould *et al.*, 2003]. In contrast, the high affinity receptor FcεRI, has a *direct* role in mediating the symptoms of allergy.

Allergen cross-links specific IgE, bound to the surface of mast cells by FcεRI in allergic tissue (and importantly for systemic anaphylaxis, also on basophils in the circulation), (*Fig.* 1.3.1). Crosslinking triggers mast cell degranulation to release histamine, cytokines, proteases, membrane-derived lipid mediators (leukotrienes and prostaglandins) and platelet-activating factor. These are responsible for the symptoms

of allergy and in combination with cytokines generated by T_H2 cells, attract inflammatory cells including macrophages and eosinophils that help constitute the late-phase response of allergic inflammation. In contrast to immediate hypersensitivity that is initiated within minutes, this late phase response induces symptoms six-nine hours after exposure to allergen [Kay, 2001b]. The degranulation of mast cells in the skin forms the basis of the skin-prick test.

Activated mast cells produce IL-4. This perpetuates the bias towards T_H2 cells present in allergic tissue, reinforced by the production of IL-4 by T_H2 cells themselves (*Fig.* 1.3.1). T_H2 production of IL-4, in combination with the expression of CD40L, up-regulated on the T cell surface in response to antigen, drives cells of other isotypes to switch to the production of IgE (see section 1.7). The importance of IL-4 in mediating the production of IgE has been illustrated by the identification of a polymorphism in the IL-4 receptor, associated with both hyper-IgE syndrome and atopy [Hershey *et al.*, 1997].

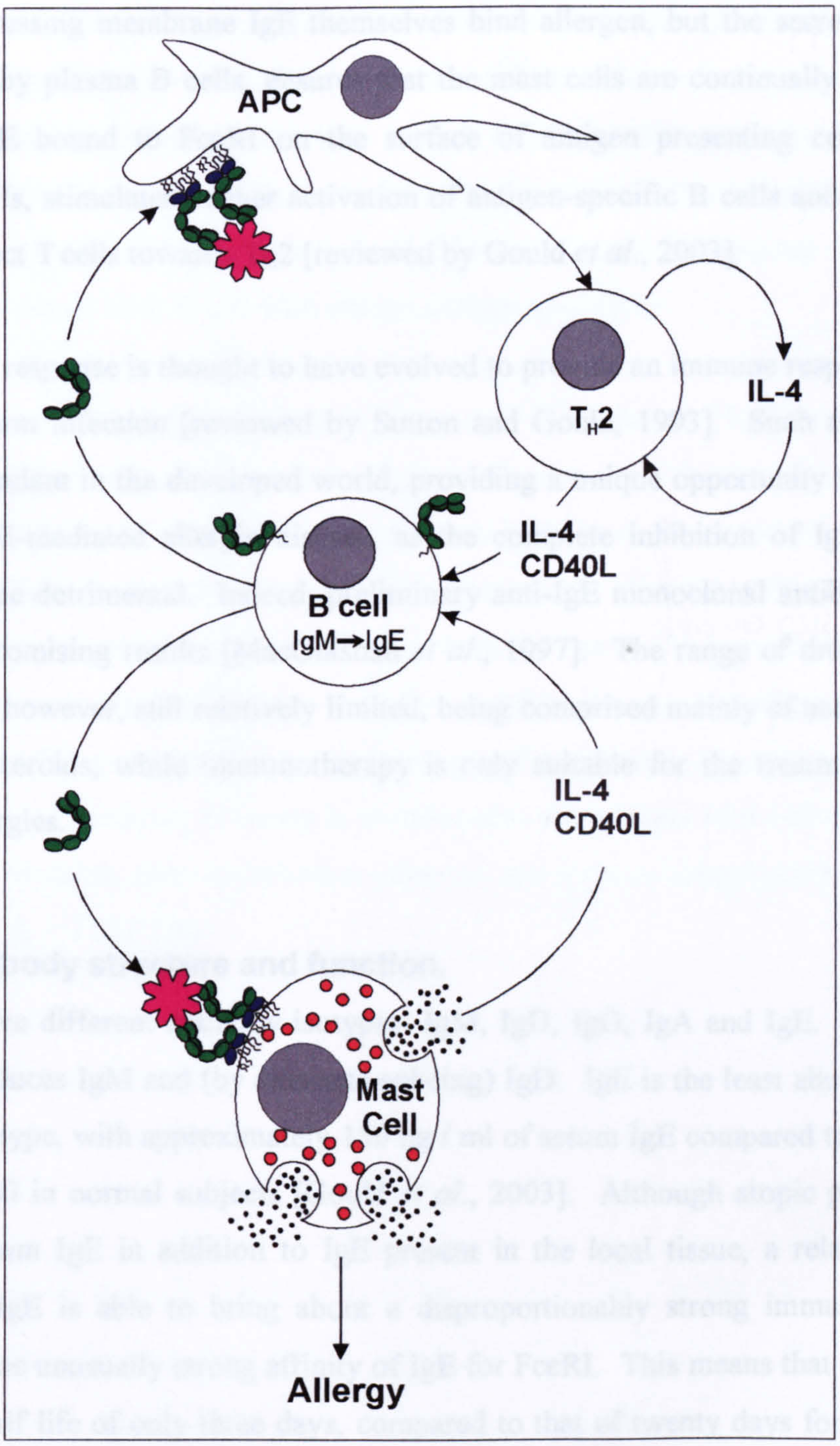


Fig.1.3.1; Interactions of the immune cells in IgE-mediated hypersensitivity. A simplified schematic demonstrates some of the important IgE mediated cellular interactions in the allergic response. B cells produce antibody that is displayed on the cell surface. The cognate interaction of allergen specific T_H2 and B cells triggers a positive feedback loop in which T cell IL-4 production is increased. This IL-4 production combined with CD40L displayed on the T cell surface stimulated B cells to switch to the production of IgE (depicted in green). IgE is secreted and also present as a membrane protein on the surface of B cells. Cells including mast cells and antigen presenting cells (APC) display soluble IgE on their cell surface via the high affinity receptor (FcεRI), (depicted in blue). The interaction of allergen (pink) with IgE on APC causes further stimulation of T_H2 cells. In addition, the IL-4 that the T cells produce reinforces the bias towards T_H2, perpetuating the allergic cycle. Crosslinking of allergen on mast cells results in their degranualtion, releasing the mediators of allergic symptoms.

B cells expressing membrane IgE themselves bind allergen, but the secretion of IgE, particularly by plasma B cells, ensures that the mast cells are continually primed. In addition, IgE bound to FcεRI on the surface of antigen presenting cells, such as dendritic cells, stimulates further activation of antigen-specific B cells and importantly can also direct T cells towards T_H2 [reviewed by Gould *et al.*, 2003].

The allergic response is thought to have evolved to provide an immune response against parasitic worm infection [reviewed by Sutton and Gould, 1993]. Such a response is mainly redundant in the developed world, providing a unique opportunity for therapies to target IgE-mediated allergic disease, as the complete inhibition of IgE would be unlikely to be detrimental. Indeed, preliminary anti-IgE monoclonal antibody therapy has given promising results [MacGlashan *et al.*, 1997]. The range of drugs currently available is, however, still relatively limited, being comprised mainly of anti-histamines and corticosteroids, while immunotherapy is only suitable for the treatment of some specific allergies.

1.4 Antibody structure and function.

There are five different antibody isotypes: IgM, IgD, IgG, IgA and IgE. Each B cell initially produces IgM and (by alternate splicing) IgD. IgE is the least abundant serum antibody isotype, with approximately 150 ng / ml of serum IgE compared to 10 mg / ml of serum IgG in normal subjects [Gould *et al.*, 2003]. Although atopic patients have elevated serum IgE in addition to IgE present in the local tissue, a relatively small amount of IgE is able to bring about a disproportionably strong immune response because of the unusually strong affinity of IgE for FcεRI. This means that while serum IgE has a half life of only three days, compared to that of twenty days for serum IgG, IgE may remain bound to FcεRI on mast cells for months [Roitt *et al.*, 1996], [reviewed by Gould *et al.*, 2003].

Class switch recombination (CSR), (described in detail in section 1.7) enables B cells activated by antigen to switch from IgM to the use, for example, of IgE. While IgE is most common in allergic tissues, IgG is mainly found in the circulation and IgA is most common in secretions. The structure of antibodies of different isotypes varies slightly, but all antibodies, regardless of isotype, have both an Fc and a Fab region (*Fig.* 1.4.1). The Fc region binds to its appropriate antibody receptor (such as FcεRI for IgE) and

includes most of the heavy chain constant region (C_H) that specifies the antibody isotype. CSR enables an antibody to bind to a different class of receptor, mediating a different effect, depending on the type of cell displaying the particular FcR and its cellular location. Even after CSR, the variable region of the Fab encoded by each B cell remains the same, as it is the Fab (specifically the variable (V) region) that binds to antigen and endows each B cell with unique antigen specificity.

All antibodies are comprised of both a heavy and light chain. The Fc region is only comprised of the heavy chain constant region, whereas the Fab comprises part of the heavy chains, the heavy chain variable region and both the constant and variable regions of the light chains (each heavy and each light chain being identical within an antibody). The interaction of antigen is generally with both the heavy (V_H) and light (V_L) chain variable regions. The V regions therefore exhibit the greatest diversity between antibodies, enabling the widest possible range of antigens to be recognised by the immune system. Antibody diversity is introduced at three stages of B cell development, V(D)J recombination, class switch recombination and somatic hypermutation (discussed in sections 1.5, 1.7 and 1.8).

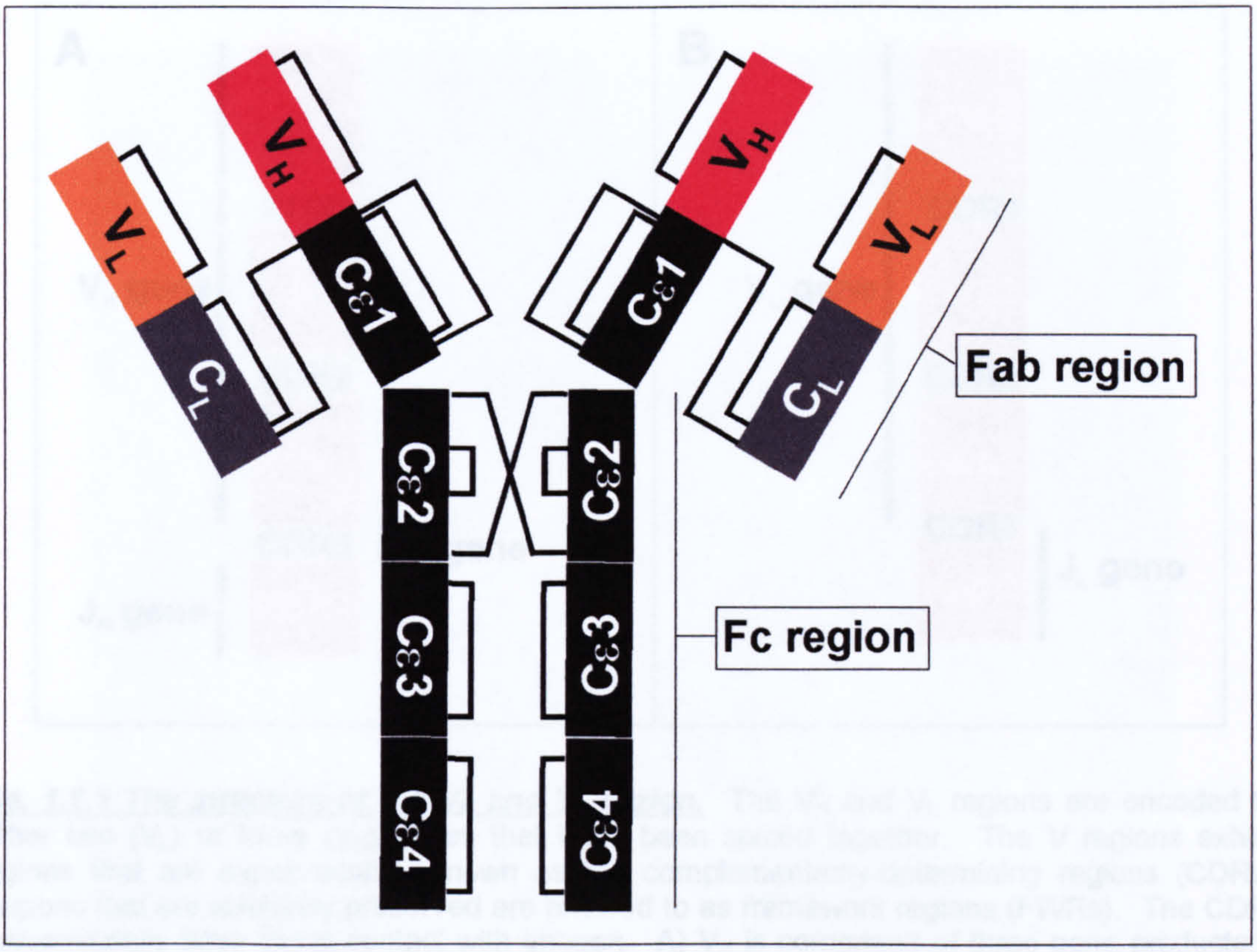


Fig. 1.4.1; The structure of IgE. IgE is comprised two identical heavy chains and two identical light chains. The different chains are held together by disulphide bonds. The heavy chain includes a variable region (V_H) and a constant region ($C\varepsilon$). The constant region of the IgE heavy chain contains four domains and specifies the effector function of the antibody. The light chain also contains both a variable region (V_L) and a constant region (C_L). In contrast however, C_L is comprised of only one domain. V_H and V_L are encoded by different repertoires of genes (see Table 1.5.1). The Fab and Fc regions are indicated.

1.5 The structure and recombination of the variable region.

Antibody heavy and light chains both have a V region, at the tip of the Fab fragment (*Fig.* 1.4.1). The V_H and V_L of each 'arm' of an antibody conventionally form an antigen binding site, such that theoretically each antibody could bind to two molecules of antigen. In order to generate a broad range of antibodies for a successful immune response, the V regions have a high level of diversity. Initially this is introduced by the recombination of different V, D (diversity) and J (joining) genes in the bone marrow.

Antibody V regions are encoded by the recombination of different V(D)J genes on human chromosome 14 (V_H) and chromosomes 2 or 22 (V_L). The V_H region is comprised of three gene products, from a V_H, a D and a J_H gene, while the V_L region is encoded by only two types of gene, V_L and J_L (*Fig.* 1.5.1).

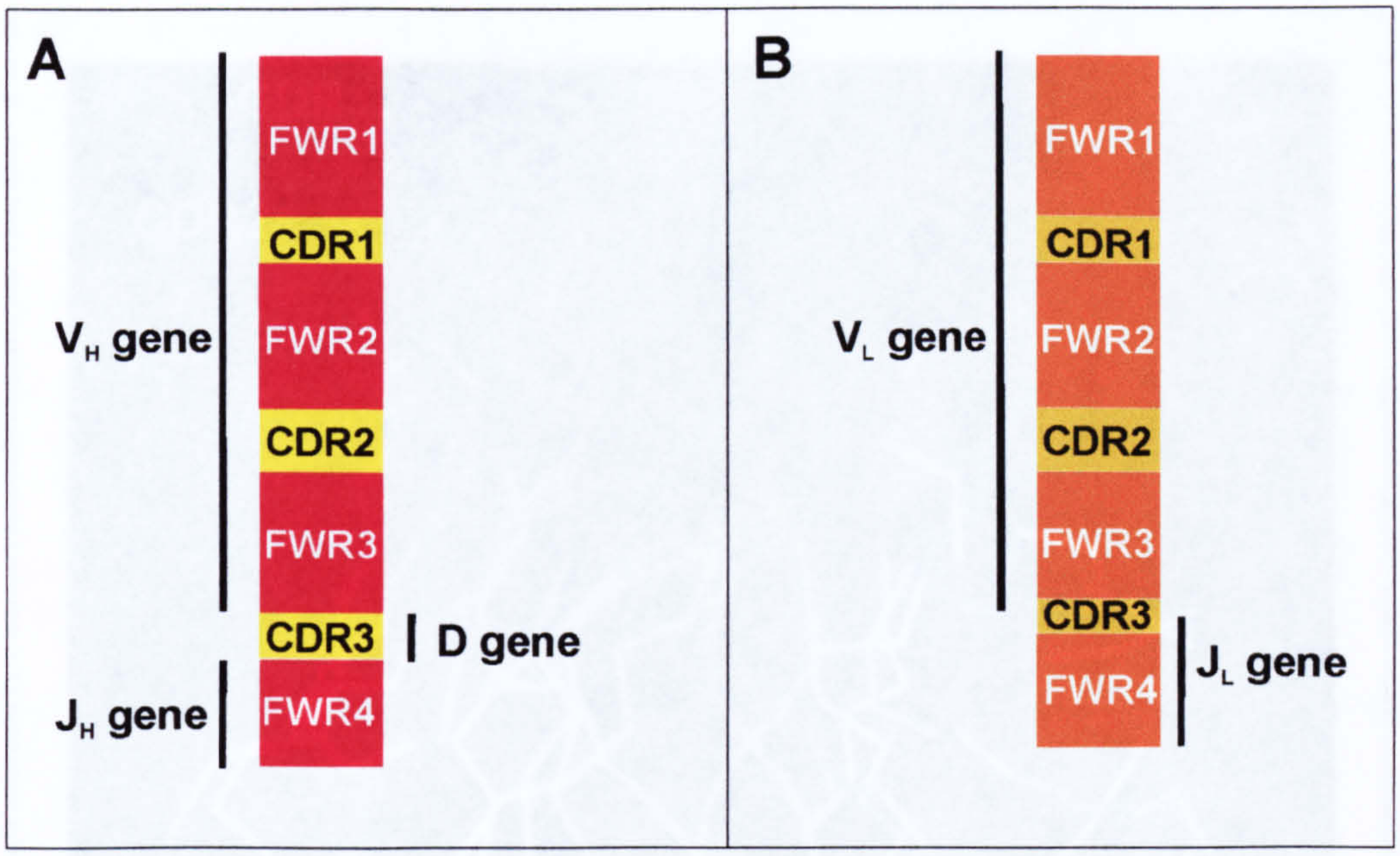


Fig. 1.5.1 The structure of the V_H and V_L region. The V_H and V_L regions are encoded by either two (V_L) or three (V_H) genes that have been spiced together. The V regions exhibit regions that are hypervariable, known as the complementarity-determining regions (CDRs). Regions that are relatively preserved are referred to as framework regions (FWRs). The CDRs conventionally have direct contact with antigen. **A)** V_H is comprised of three gene products; a V_H gene encodes FWR1, 2 and 3, CDR1 and 2, while a D gene encodes CDR3. FWR4 is encoded by a J_H gene. **B)** In contrast, V_L is encoded by only two genes, a V_L gene encodes FWR1, 2 and 3, CDR1 and 2, but there is no D gene. CDR3 is encoded by the junction of V_L and J_L. The J_L genes also encode FWR4.

Within the V regions, the complementarity determining regions (CDRs), conventionally have direct contact with antigen by forming loops at the tip of the antibody (*Fig.* 1.5.2). Of the CDR regions, CDR1 and CDR2 are encoded by a V gene. CDR3 is encoded primarily by D in the heavy chain, but by the V-J junction in the light chain. In contrast, framework regions (FWRs) do not generally have a direct role in antigen binding, but are necessary for the structural stability of the antibody. However, the sequence of the FWRs can affect the conformation and position of the CDRs [Tramontano *et al.*, 1990]. FWR1, 2 and 3 are encoded by the V gene, while FWR4 is encoded by the J gene.

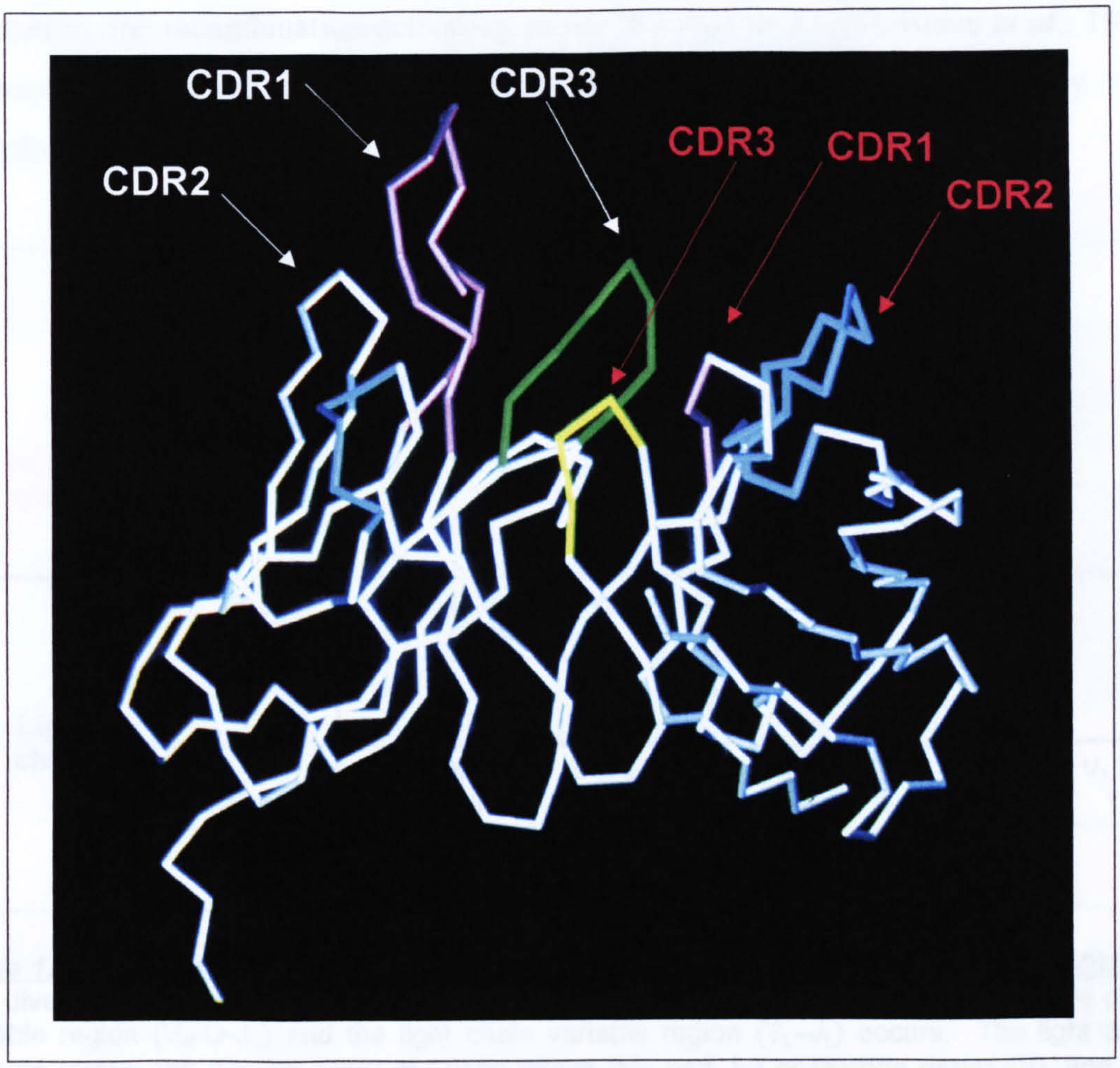


Fig. 1.5.2; The CDR loops of the antibody heavy and light chain [courtesy of B. Sutton].
The Cα backbone of the heavy and light chain variable regions illustrates the CDR loops. On the left of the figure the position of CDR1, 2 and 3 of the heavy chain is detailed (labelled in white). On the right of the figure the position of CDR1, 2 and 3 of the light chain is detailed (labelled in orange).

The V, D and J genes are taken from such a large repertoire that they can be divided into gene classes, in which the sequences have approximately 80% homology (Table 1.5.1). The choice of which V (D) or J gene is recombined with the other is random. However, each gene has a recombination signal sequence (RSS) that includes either a 12 or 23 bp spacer sequence. The '12 / 23 bp rule' dictates that genes are only able to recombine if their RSSs have different length spacers [reviewed by Lewin, 2000]. In the mouse, for example, the V_H genes are each followed by a RSS that includes a 23 bp spacer. The D genes are both preceded and followed by 12 bp spacers, while each J_H gene is preceded by a 23 bp spacer [Lewin, 2000]. This ensures that a V_H gene does not join directly to a J_H gene. Breakage of the chromosome during recombination is

directed by the recombination-activating genes (RAG) 1 and 2 [McBlane *et al.*, 1995]. Subsequent repair of the rearranged chromosome is thought to occur by non-homologous end-joining [reviewed by Grawunder and Harfst, 2001].

	Total number of genes	Numbers of V, D and J genes in each class									
Heavy chain	V_H 51	V_H1 11	V_H2 3	V_H3 22	V_H4 11	V_H5 2	V_H6 1	V_H7 1			
	D 25	D1 4	D2 4	D3 5	D4 4	D5 4	D6 3	D7 1			
	J_H 6	J_H1 1	J_H2 1	J_H3 1	J_H4 1	J_H5 1	J_H6 1				
Light chain	Vκ 40	$V_κ1$ 19	$V_κ2$ 9	$V_κ3$ 7	$V_κ4$ 1	$V_κ5$ 1	$V_κ6$ 3	$V_κ7$ 0			
	Jκ 5	$J_κ1$ 1	$J_κ2$ 1	$J_κ3$ 1	$J_κ4$ 1	$J_κ5$ 1					
	Vλ 31	$V_λ1$ 5	$V_λ2$ 5	$V_λ3$ 9	$V_λ4$ 3	$V_λ5$ 3	$V_λ6$ 1	$V_λ7$ 2	$V_λ8$ 1	$V_λ9$ 1	$V_λ10$ 1
	Jλ 4	$J_λ1$ 1	$J_λ2$ 1	$J_λ3$ 1	$J_λ4$ 0	$J_λ5$ 0	$J_λ6$ 0	$J_λ7$ 1			

Table 1.5.1 The repertoire of functional human V, D and J genes [Adapted from VBase].
The diverse repertoire of V, D and J genes from which recombination to form the heavy chain variable region (V_H-D-J_H) and the light chain variable region (V_L–J_L) occurs. The light chain variable region can consist either of kappa genes (Vκ and Jκ) or lambda genes (Vλ and Jλ). The genes are divided into classes on the basis of sequence similarities (with greater than 80% homology between the sequences in each class). Only the genes known to have an open-reading frame and therefore be functional are detailed. Those classes that have 0 members, are constituted solely of non-functional genes.

In the pro-B cell the V_H region is the first to be rearranged. Initially a D and J_H gene are brought together, then rearrangement of a V_H gene occurs such that V_H, D and J_H can encode a continuous mRNA transcript (*Fig.* 1.5.3). If the heavy chain V_H-D-J_H rearrangement enables the production of a functional protein, then the light chain is also subject to rearrangement. If the genes have been rearranged such that the reading frame is no longer in-frame, then heavy chain rearrangement on the second chromosome takes place. If this is also non-productive, the cell is subject to apoptosis [Janeway et al., 2000].

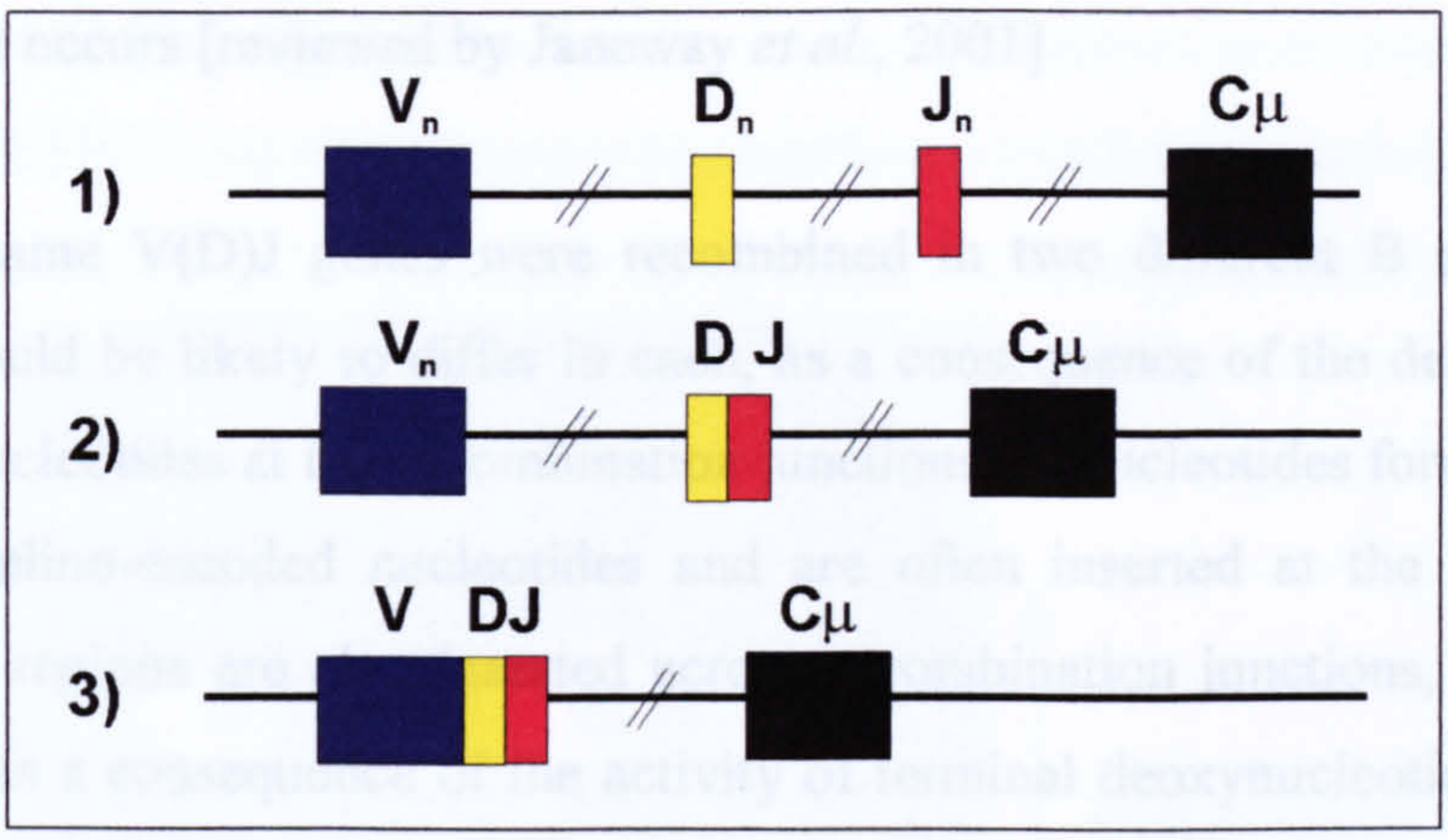


Fig. 1.5.3 The order of heavy chain VDJ rearrangement. A simplified schematic demonstrates the order in which the V (variable), D (diversity) and J (joining) genes rearrange on chromosome 14. Initially one of the D genes is recombined with one of the J_H genes. Then one of the V_H genes is recombined with DJ_H. In the first instance Cμ is proximal, resulting in the expression of IgM if rearrangement of the light chain is also productive.

The light chain can either be kappa (κ), or lambda (λ) in origin, so that the light chain is comprised of either Vκ-Jκ-Cκ or Vλ-Jλ-Cλ. Initially in the pre-B cell, rearrangement of the kappa locus takes place on chromosome 2. If this is unproductive, then recombination of the kappa locus on the second chromosome takes place. The Vκ genes are clustered upstream of the Jκ genes. This means that on each kappa chromosome there are multiple opportunities for correct rearrangement. For example, if initially an unproductive out-of-frame rearrangement occurs between a 3' Vκ gene and a 5' Jκ gene, it is possible for recombination to be attempted again on the same chromosome. In this instance a more 5' Vκ gene would recombine with a more 3' Jκ gene, excising the intervening out-of-frame Vκ-Jκ segment [reviewed in Lewin, 2000 and Janeway *et al.*, 2000]. As a consequence, in humans the majority of antibodies are kappa [Foster *et al.*, 1997].

If all the opportunities for kappa rearrangement fail, rearrangement of the lambda locus occurs on first one and then if necessary, the other chromosome 22. However, in contrast to the kappa chromosome, multiple opportunities to rescue an unproductive lambda locus are not thought to arise. If rearrangement of both the kappa and then the lambda light chain are unsuccessful, the cell is subject to apoptosis. On both the heavy

and light chain chromosomes, allelic exclusion ensures that only one productive rearrangement occurs [reviewed by Janeway *et al.*, 2001]

Even if the same V(D)J genes were recombined in two different B cells, antibody specificity would be likely to differ in each, as a consequence of the deletion and / or insertion of nucleotides at the recombination junctions. P nucleotides form palindromes with the germline-encoded nucleotides and are often inserted at the recombination junctions. N regions are also inserted across recombination junctions, mainly in the heavy chain, as a consequence of the activity of terminal deoxynucleotidyl transferase [Desiderio *et al.*, 1984]. These also consist of non-germline encoded nucleotides, but are of random sequence. The insertions and deletions that are introduced across the V_H-D-J_H junction results in CDR3 having the greatest diversity of all the CDRs. CDR3 is therefore regarded as a 'signature region', unique to each B cell clone and provides a method by which descendents of the same progenitor cell can be identified.

1.6 Sites of B cell maturation.

After productive V(D)J recombination of a B cell in the bone marrow, IgM and (by alternate splicing) IgD are expressed. The B cell is considered to be mature, although naive, as it has not yet encountered antigen. When a mature, naive B cell is released from the bone marrow it enters the circulation and migrates towards a site of secondary lymphoid tissue. These include the spleen, lymph nodes, gut-associated lymphoid tissue and mucosal-associated lymphoid tissue. In this secondary lymphoid tissue, B cells encounter antigen. Antigen is usually presented by dendritic cells after acquisition at a peripheral site. B cell activation is triggered by exposure to antigen in the presence of an antigen specific T cell.

Further maturation of a B cell is dependent upon successful class switch recombination (CSR) to change the isotype of the antibody, and somatic hypermutation (SHM), in which mutation of the V region sequences leads to increased affinity of the antibody for its antigen. CSR and SHM both require the expression of activation-induced cytidine deaminase (AID) [Muramatsu *et al.*, 2000] and all three occur in germinal centres [Liu *et al.*, 1996], [Jacob *et al.*, 1991], [Kuppers *et al.*, 1993], [Muramatsu *et al.*, 1999]. Activated B cells migrate towards, or initiate, a germinal centre within the secondary

lymphoid tissue. Initially, an activated B cell enters the dark zone of a germinal centre (*Fig.* 1.6.1). SHM is triggered as B cells develop to become centroblasts [Pascual *et al.*, 1994], both SHM and clonal expansion therefore occurring in the dark zone.

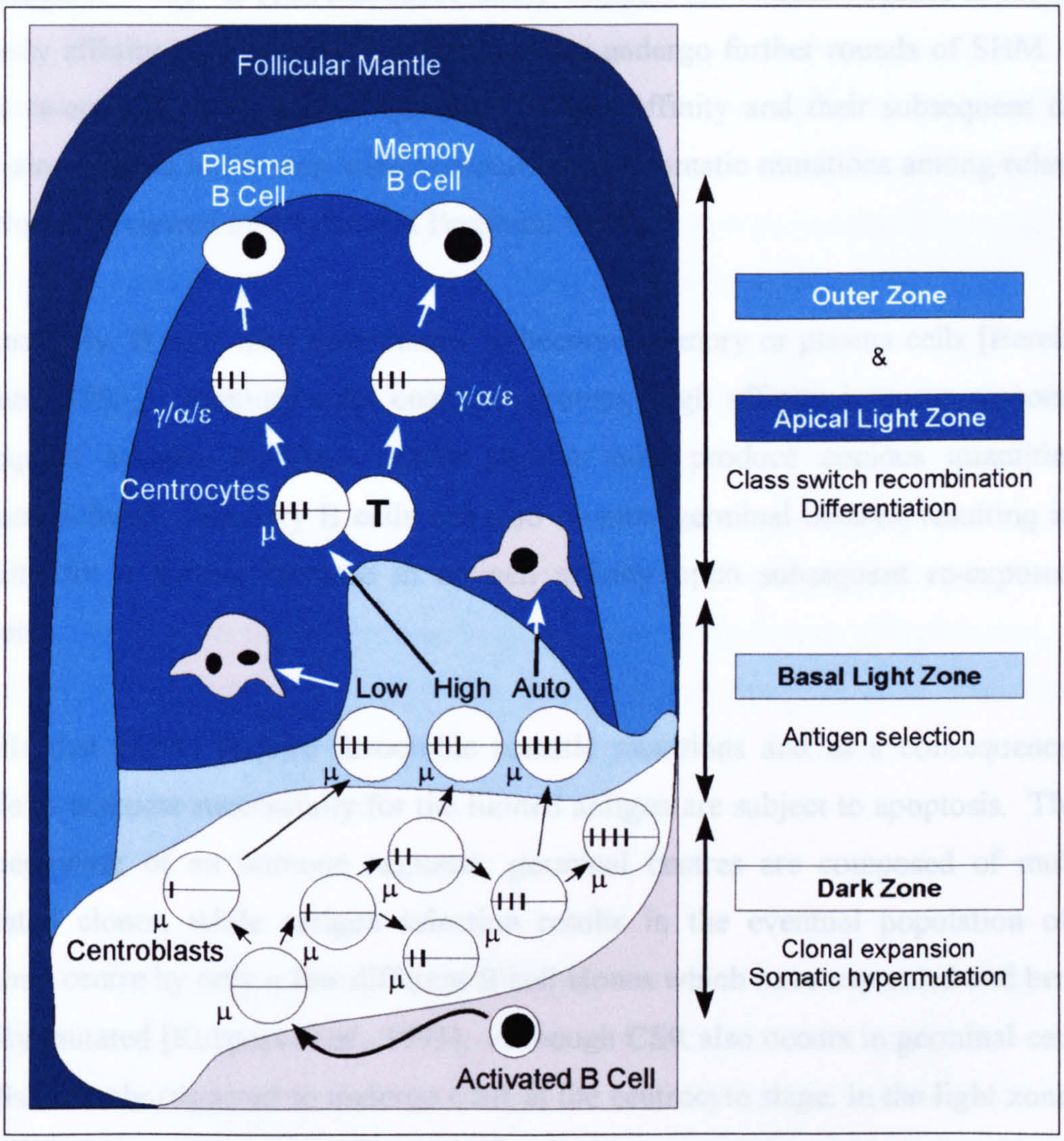


Fig. 1.6.1; A schematic demonstrating the migration of a B cell through the germinal centre [adapted from Liu et al., 1996]. Initially, an activated B cell enters the dark zone of the germinal centre, becoming a larger centroblast. Centroblasts are subject to somatic hypermutation and clonal expansion. The B cells then migrate into the light zone, where they become centrocytes that display the mutated antibody on their surface. In the basal light zone these mutated antibodies are subject to antigen selection by antigen displayed on follicular dendritic cells. B cells of high affinity progress to the apical light zone and outer zone, where the presence of T cells is thought to trigger CSR and further clonal expansion. B cells then differentiate into either memory or plasma B cells. Alternatively, they may re-enter the germinal centre dark zone (hypothesised by [Kepler and Perelson, 1993] and not detailed in this figure).

After affinity maturation by SHM, clones progress to the light zone where they are exposed to antigen displayed by follicular dendritic cells (FDC's). Increased affinity for this antigen results in consequential competition between B cells [reviewed by Berek and Ziegner, 1993]. B cells that successfully compete for antigen because of increased antibody affinity may re-enter the dark zone to undergo further rounds of SHM. The cyclic re-entry of B cells with increased antigen affinity and their subsequent clonal expansion, results in the step-wise accumulation of somatic mutations among related B cell clones [reviewed by Kepler and Perelson, 1993].

Alternatively, B cells may differentiate to become memory or plasma cells [Berek and Ziegner, 1993]. Memory cells enable a prompt, high affinity immune response to subsequent antigen challenge, while plasma cells produce copious quantities of immunoglobulin. Memory B cells can also re-enter germinal centres, resulting in the capacity for a further increase in antigen affinity upon subsequent re-exposure to antigen.

B cells that fail to acquire favourable somatic mutations and as a consequence are unable to compete successfully for the limited antigen are subject to apoptosis. Thus at the beginning of an immune response, germinal centres are composed of multiple unrelated clones, while antigen selection results in the eventual population of the germinal centre by only a few different B cell clones which have expanded and become heavily mutated [Kuppers *et al.*, 1993]. Although CSR also occurs in germinal centres, B cells are only triggered to undergo CSR at the centrocyte stage, in the light zone and outer zone [Liu *et al.*, 1996], where T cells are able to provide cytokines (*e.g.* IL-4) and also CD40L necessary to instigate CSR.

Although CSR and SHM were conventionally assumed to be confined to the secondary lymphoid tissue, it has been demonstrated that when chronic antigen stimulation occurs, these processes may occur in other tissues. This has most frequently been demonstrated in autoimmune diseases. In ankylosing spondylitis, germinal centres have been shown to develop abnormally in the synovial membrane [Voswinkel *et al.*, 2001] and have also been identified in the pancreas of the murine model of diabetes mellitus [Ludewig *et al.*, 1998].

Research conducted on a murine model of autoimmunity suggested that somatic hypermutation in the spleen was able to occur outside of germinal centres [William *et al.*, 2002]. However, the presence of local germinal centre-like structures in the synovial membranes of rheumatoid arthritis patients has been researched in greatest detail. Repeated analysis has suggested that these germinal centre-like structures contain B cells, T cells and FDC's [Randen *et al.*, 1995], [reviewed by Berek and Kim, 1997]. Although not occurring in lymphoid tissue, these B cells have been demonstrated to undergo SHM, CSR and clonal expansion as an apparent consequence of persistent local antigen [Schroder *et al.*, 1996], [Williams *et al.*, 1999].

Germinal centre formation was also stimulated to occur in the murine lung after antigen challenge [Chvatchko *et al.*, 1996]. In contrast to rheumatoid arthritis, these germinal centres did occur within secondary lymphoid tissue (bronchial-associated lymphoid tissue, BALT). They generated antigen-specific IgE^+ plasma cells. It was therefore suggested that the chronic antigen stimulation observed in *human* allergic asthma may also stimulate local germinal centre-like structures and therefore local production of IgE^+ memory and plasma cells [Chvatchko *et al.*, 1996]. To date however, no such observations have been reported.

1.7 The induction of IgE production by class switch recombination.

In order for a B cell to be induced to switch isotype to IgE in the germinal centre, it must receive two signals, the first from IL-4 (or IL-13) and the second *via* the interaction of CD40 with its ligand [Jabara *et al.*, 1990], [Zhang *et al.*, 1991], [Shapira *et al.*, 1992], [Armitage *et al.*, 1993]. Both signals are provided by T_H2 cells, but IL-4 is additionally produced by allergen stimulated mast cells (and basophils in the blood). IL-4 stimulates the induction of ε germline transcription (IL-13 also has this ability and works *via* a similar mechanism to IL-4).

The binding of IL-4 to its receptor (IL-4R), on the B cell surface, triggers a signalling cascade that ultimately stimulates ε germline transcription (reviewed by [Nelms *et al.*, 1999], [Oettgen, 2000] and [Gould et al., 2000]). Interaction of IL-4 with IL-4R results in the activation of the JAK1 and JAK3 kinases, these subsequently phosphorylate the transcription factor STAT-6. Iε, the ε germline promotor, includes a STAT-6 binding

site. In order for successful ε germline gene transcription to be stimulated however, two other transcription factors, NFκB and BSAP, must also be present.

Expression of both NFκB and BSAP are stimulated by the CD40-CD40L interaction [Gould *et al.*, 2000], [Thienes *et al.*, 1997]. The CD40-CD40L interaction is also necessary for B cell expression of CD80 and B cell proliferation [reviewed by Gould *et al.*, 2000]. The interaction of CD80 with CD28 (on T cells) forms a positive feedback loop whereby further synthesis of IL-4 is stimulated.

Germline gene transcription is essential for CSR, because in order for a different heavy chain constant gene (C_H) to be used, rearrangement and therefore chromosomal breaks must occur. In order for a B cell to switch from its initial production of IgM, to the production of IgE for example, both the μ (IgM) and ε (IgE) locus must be actively transcribed. The heavy chain genes are arranged in tandem on chromosome 14 (*Fig.* 1.7.1). The arrangement of chromosome 14 is such that every C_H gene (with the exception of IgD) is preceded by a promotor (I) and also a switch region (S).

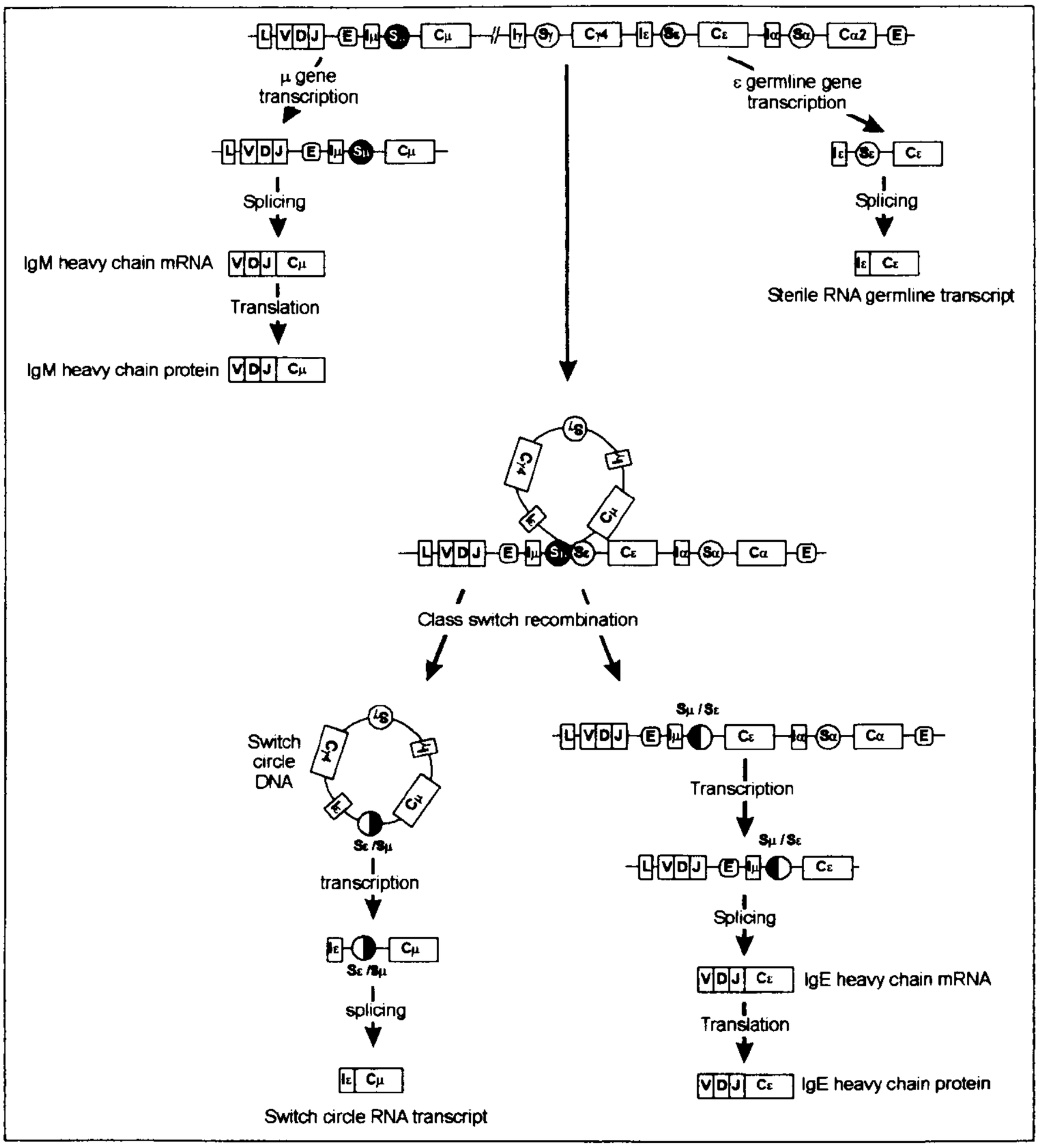


Fig. 1.7.1; Class switch recombination [adapted from Gould et al., 2003]. Class switch recombination (CSR) enables the isotype that an antibody expresses to be changed by the repositioning of the heavy chain constant region (C_H) genes encoded by chromosome 14. The C_H genes ordered IgM (Cμ), IgD (Cδ), IgG3 (Cγ3), IgG1(Cγ1), IgA1(Cα1), IgG2 (Cγ2), IgG4 (Cγ4), IgE (Cε), then finally IgA2(Cα2). In this figure, only Cμ, Cγ4 and Cα2 are detailed. Each C_H gene (except Cδ) is preceded by a promoter (I) and a switch region (S). Gene transcription occurs at both of the loci to be rearranged, producing a sterile germline transcript at the donor locus, from which the S region is excised (see text for details). CSR (in this instance from IgM to IgE) generates a switch circle of excised DNA that is transiently transcribed. Transcription of the rearranged heavy chain, leads to the expression of IgE. The position of the leader (L), variable (V), diversity (D) and joining (J) regions and enhancers (E) are also detailed.

Germline gene transcription occurs across I, S and C_H. Therefore ε germline transcripts are comprised Iε-Sε-Cε (*Fig.* 1.7.1). Splicing of the germline transcripts means that Sε is removed, resulting in Iε-Cε. The I exons contain a stop codon in each of the three reading frames. This means that all germline transcripts are sterile. Class switch

recombination is thought to rely on the excised S region enabling the formation of an R loop (*Fig.* 1.7.2).

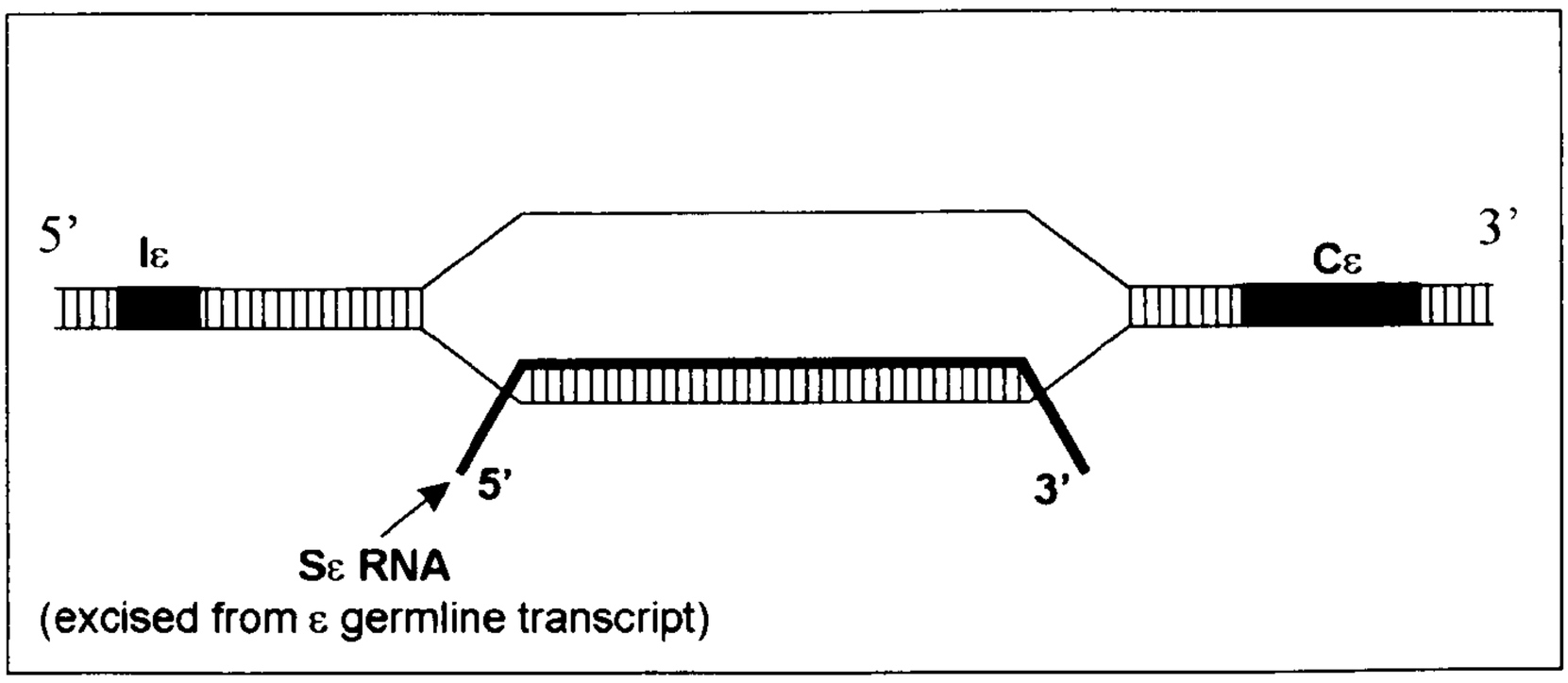


Fig.1.7.2; R loop formation. The formation of an R loop takes place as a consequence of germline gene transcription of a C_H locus on chromosome 14 and enables class switch recombination to take place. In an IgM to IgE switch, for example, trancripts are produced from both Cμ and Cε. The S region is excised from the transcripts. In the case of the Cε locus, the Sε RNA remains paired to the Sε DNA, forming an R loop of single stranded DNA. The DNA at both the Cμ and Cε locus is then cleaved by an AID mediated mechanism, enabling CSR to take place as illustrated in *Fig.* 1.7.1.

At both the Sμ and Sε sites actively undergoing transcription, the excised S region mRNA forms a DNA-RNA hybrid with the genomic S region DNA, forming an R loop. It has been suggested that the donor and acceptor switch sites are held in close proximity by trans-spliced transcripts *i.e.* a transcript that includes the 5' end of Sμ and the 3' of Sε [Fujeida *et al.*, 1996]. It is thought that the DNA is cleaved at the end of the R loop, where the DNA is single stranded [Tian and Alt, 2000]. Ongoing research into the exact mechanism of CSR has suggested that AID may mediate these DNA breaks [Petersen *et al.*, 2001], [Petersen-Mahrt *et al.*, 2002], which may be targeted to secondary structures in the single stranded DNA [Kinoshita and Honjo, 2001], [Chen *et al.*, 2001]. (see section 1.10). The DNA breaks enable CSR between the two C_H regions before the DNA is repaired by non-homologous end-joining [reviewed by Honjo *et al.* 2002].

The seemingly clear sequence of events by which IL-4 mediates ε germline transcription is confused by recent findings that a single B cell, when incubated *in vitro* with IL-4 and αCD40, is in fact stimulated to produce germline transcripts of multiple

different isotypes [David Fear and Hannah Gould, unpublished observations]. What finally determines that the resultant switch, stimulated by IL-4 and αCD40, is to IgE is therefore yet to be determined.

The process of CSR not only enables direct switching of IgM to IgE, for example, but also indirect switches *e.g.* from IgM to IgG and then to IgE, as a consequence of multiple CSR events. This has been identified by the analysis of switch circles, the loop of genomic DNA excised when the two heavy chain genes are brought together (*Fig.* 1.7.1) [Zhang *et al.*, 1994]. Switch circle DNA is therefore often used as a marker of CSR, although it is not thought to degrade quickly, but rather be diluted by cell division. A recent discovery that transcription of the switch circle DNA occurs for a limited amount of time, has led to the use of switch circle transcripts as a marker of recent CSR [Kinoshita *et al.*, 2001].

In addition to stimulation by IL-4 and CD40L, CSR is also though to be dependent upon the number of cell divisions that the B cell has undergone. Experiments on murine B cells suggest that in the presence of IL-4 and αCD40, ε germline transcription is stimulated after four cell divisions and CSR to IgE after five cell divisions. Germline transcription and CSR to other isotypes was observed to occur after fewer cell divisions [Hasbold *et al.*, 1998], [McCall and Hodgkin, 1999].

1.8 Somatic hypermutation.

Somatic hypermutation (SHM) is the process by which the affinity of an antibody is increased, enabling the body to mount a more efficient immune response. As detailed in section 1.6, SHM only occurs in B cells that have been activated by the presence of both antigen and T cells and in normal circumstances takes place in the germinal centre dark zone, where B cells are present as centroblasts.

Clonal expansion in the dark zone results in a population of closely related B cells that are distinguished by the different somatic mutations that they have accumulated. Competition between the B cells for a limited quantity of antigen, presented by follicular dendritic cells, occurs in the light zone of the germinal centre, where the B cells are present as centrocytes. Those B cells exhibiting mutations that increase their

affinity for antigen compete most successfully for antigen and may re-enter the germinal centre to undergo further rounds of SHM, or differentiate to become memory or plasma B cells [reviewed by Berek and Ziegner, 1993].

This results in the accumulation of B cells that while related (as determined by an identical CDR3 sequence rearrangement), differ in the mutations they have acquired (and possible as a result of CSR, their isotype), but which can be positioned in a genealogical tree because of the step-wise accumulation of somatic mutations. In such a genealogical tree, mutations acquired early in the maturation of the B cell will be shared by the greatest number of sister clones, while each B cell will also exhibit mutations unique to its most recent round of SHM.

Somatic mutation occurs at a rate of approximately 10^{-3} base pairs per generation [McKean *et al.*, 1984], [Berek and Milstein, 1987]. Antibody affinity generally increases in response to an increased number of mutations, however, the introduction of just one mutation has been demonstrated to be capable of increasing antibody affinity by a factor of 10 [Berek and Milstein, 1987].

Somatic mutations usually take the form of single nucleotide substitutions (with deletions and insertions occurring rarely). These mutations are specifically introduced into V_H and V_L DNA in both productive and non-productively rearranged loci. Un-rearranged genes are not generally mutated [reviewed by Neuberger and Milstein, 1995]. Somatic mutations are confined to approximately 2 kb surrounding the V region, such that mutations occur as far 5' as the promoter and as far 3' as the intron between the J gene and the constant gene [Lebecque and Gearhart, 1990]. The level of mutation follows a gradient, with the greatest number of mutations occurring in the V region itself, less in J and none in C. Bcl-6 is also subject to SHM, but at a lower level than the V region [Shen *et al.*, 1998], [Pasqualucci *et al.*, 1998].

The introduction of a mutation may have a silent effect on the amino acid that is encoded, or alternatively may result in an amino acid replacement. Only replacement mutations alter the affinity of an antibody. Mutations are referred to as non-intrinsic if they are a consequence of antigen selection. Even in the absence of antigen selection, SHM is not random. The intrinsic preferences of SHM were determined by the analysis

of passenger transgenes and sequences that flanked the V region and used to generate a summary of the preferences of SHM for particular bases (see appendix C) [Betz *et al.*, 1993b]. SHM has been suggested to be targeted to one DNA strand, the most obvious consequence being the biased occurrence of an A→T as opposed to a T→A mutation [Lebecque and Gearhart, 1990], [Betz *et al.*, 1993b], [Insel and Varade, 1994], [Rogozin *et al.*, 2001].

Mutations that result in a transition (the replacement of one purine (A or G) with another, or one pyrimidine (C or T) with another) are more common than mutations that result in a transversion (the replacement of a purine with a pyrimidine, or *vice versa*) [Insel and Varade, 1994]. In addition, certain DNA sequence motifs have been demonstrated to be hotspots for SHM [reviewed by Rogozin and Pavlov, 2003]. These include R<u>G</u>YW, its reverse complement, WR<u>C</u>Y and W<u>A</u> (where <u>G</u> or <u>C</u> are mutated and R = purine, Y = pyrimidine and W = A or T) [Rogozin and Kolchanov, 1992], [Rogozin *et al.*, 2001]. In contrast, some residues are rarely targeted for mutation and are therefore regarded as cold spots.

Even if a mutation does not adhere exactly to the R<u>G</u>YW motif, it is more likely to occur at a position that exhibits a similar motif [Rogozin and Pavlov, 2003]. In some instances a mutation may occur at a hotspot motif but not increase antibody affinity. However, if the overall mutational load of the antibody has a favourable effect on affinity, selection means that such mutations are perpetuated. These are referred to as intrinsic mutations and reflect the transition / transversion bias and the other intrinsic preferences of SHM.

Within the V region, the complementarity determining regions (CDRs) are particularly heavily mutated [Insel and Varade, 1994]. It is thought that the CDRs evolved to exhibit a greater mutability because of their direct interaction with conventional antigens and therefore importance in antibody affinity. The V_H CDRs are composed of sequences that are inherently more mutable [Chang and Casali, 1994]. This is evident by the presence of a bias towards the AGY serine codon, as opposed to the TCN serine codon, with only AGY conforming to the R<u>G</u>Y hotspot motif [Wagner *et al.*, 1995]. However, not all mutations occur in the CDRs, indeed some mutations in the FWRs have a positive effect on antigen affinity, either because the amino acid is at the edge of

the antigen binding site created by the CDRs, or because the FWR residue has an indirect affect upon the structure adopted by the CDRs [Tramontano *et al.*, 1990], [Jolly *et al.*, 1996].

The mechanism of SHM is not fully understood, although it is being elucidated as a consequence of the identification of AID. AID has been demonstrated to be required for SHM [Muramatsu *et al.*, 1999], [Muramatsu *et al.*, 2000]. Indeed, the induction of AID expression has been shown to artificially stimulate SHM in fibroblasts [Okazaki *et al.*, 2002]. The latest thinking suggests that AID acts directly on V region DNA, deaminating cytosine to form uracil. The uracil is excised, generating a break in the DNA, whose repair introduces an error(s).

The break that occurs in the DNA may be either single stranded, or double stranded if two cytosines on opposite DNA strands are deaminated (generating a staggered break) [Sale and Neuberger, 1998], [Papavasiliou and Schatz, 2000], [Bross *et al.*, 2000]. These DNA breaks have been demonstrated to correspond to RGYW motifs [Bross *et al.*, 2000].

To date, DNA polymerase ζ, ι, and η have been implicated in the error-prone repair [Zan *et al.*, 2001], [Faili *et al.*, 2002], [Rogozin *et al.*, 2001], [Zeng *et al.*, 2001], [Yavuz *et al.*, 2002]. In particular, Pol η appeared to generate mutations that correlated with the WA hotspot motif and T/A bias [Rogozin *et al.*, 2001], [Zeng *et al.*, 2001], [Yavuz et al., 2002]. However the occurrence of mutations at RGYW / WRCY appeared to be equally distributed on both DNA strands, suggesting that these effects were mediated separately [Rogozin *et al.*, 2001], [Zeng *et al.*, 2001], (see section 1.9).

Even when the V region DNA is substituted, for example by β-globin, SHM still occurs [Yelamos *et al.*, 1995]. The presence of a promotor (although not necessarily the V region promotor) and therefore transcription is necessary for somatic mutation [Betz *et al.*, 1994], [Peters and Storb, 1996] although the full degree of mutation is dependent upon the presence of the V region enhancers [Betz *et al.*, 1994]. This may correlate with the necessity of transcription for cytidine deamination by AID and it has been proposed that the hotspots of SHM may be a consequence of the secondary structure

that the single stranded DNA may adopt while transcription takes place (see section 1.9).

1.9 Activation-induced cytidine deaminase.

Until recently, there was little information as to how either class switch recombination or somatic hypermutation were coordinated. Activation-induced cytidine deaminase (AID) was then demonstrated to be required for both processes [Muramatsu *et al.*, 1999], [Muramatsu *et al.*, 2000]. Consistent with this observation, AID expression was identified in germinal centre B cells [Muramatsu *et al.*, 1999] and lymphoid tissue [Muramatsu *et al.*, 2000], [Muto *et al.*, 2000]. In addition, AID was also shown to be required for gene conversion [Arakawa *et al.*, 2002], [Harris *et al.*, 2002b], the process by which diversity is introduced into V genes in chickens, rabbits, cattle and pigs (as opposed to, or in addition to SHM) [reviewed by Janeway *et al.*, 2000].

The importance of AID in the maturation of the immune response was reinforced by the observation that a subset of patients suffering from hyper-IgM syndrome (HIGM2), displayed mutations in AID that down-regulated or abrogated its expression [Revy *et al.*, 2000], [Minegishi *et al.*, 2000]. As a consequence, these patients were subject to repeated infection, had enlarged germinal centres, severe depletion of antibody isotypes other than IgM and a lack of SHM.

AID is a member of the APOBEC family of deaminases and has homology to APOBEC-1 [Muramatsu *et al.*, 1999]. Although members of the APOBEC family have been demonstrated to be able to deaminate DNA [Harris *et al.*, 2002], [Petersen-Mahrt and Neuberger, 2003], *in vivo* APOBEC-1 deaminates C→U in apoB RNA, introducing a premature stop codon. This RNA editing generates two functional proteins (apoB100 and apoB48) with different functions [Innerarity *et al.*, 1996]. AID was also shown to exhibit cytidine deaminase activity [Muramatsu *et al.*, 1999]. However, AID was not capable of deaminating RNA [Muramatsu *et al.*, 1999], but was shown to act on DNA [Petersen-Mahrt *et al.*, 2002], [Di Noia and Neuberger, 2002].

More specifically, AID has been shown to act on single stranded DNA and correlating with CSR and SHM, AID activity is also dependent upon transcription [Ramiro *et al.*,

2003], [Chaudhuri *et al.*, 2003], [Sohail *et al.*, 2003], [Bransteitter *et al.*, 2003], [Dickerson *et al.*, 2003], [Pham *et al.*, 2003]. It has been suggested that mutations induced by AID are favoured at some positions (hotspots) as a consequence of the secondary structure that the single stranded DNA might adopt whilst undergoing transcription. It has been suggested that stem-loop structures, prone to deamination, may transiently occur in V region DNA whilst it is transcribed and also in the single stranded regions of S region DNA in the R loop during CSR [Kinoshita and Honjo, 2001], [Chen *et al.*, 2001].

AID was demonstrated to deaminate DNA from C→U when expressed in *E. coli*. Mutations introduced as a consequence of AID expression were enhanced when uracil-DNA glycosylase (UNG) deficient *E. coli were* used [Petersen-Mahrt et al., 2002] and the trends in SHM distorted in both the DT40 (chicken) cell line in which UNG was inhibited and also in UNG deficient mice, [Di Noia and Neuberger, 2002], [Rada *et al.*, 2002].

A multi-phase mechanism was suggested to account for the characteristic traits observed in SHM, CSR and UNG deficiency [Petersen-Mahrt *et al.*, 2002], (see *Fig.* 1.9.1). *Usually* dU is removed from DNA by base excision repair, creating an abasic site, which when subsequently removed by an apyrimidinic-endonuclease, causes a nick in the DNA. The DNA is then correctly repaired by a DNA polymerase.

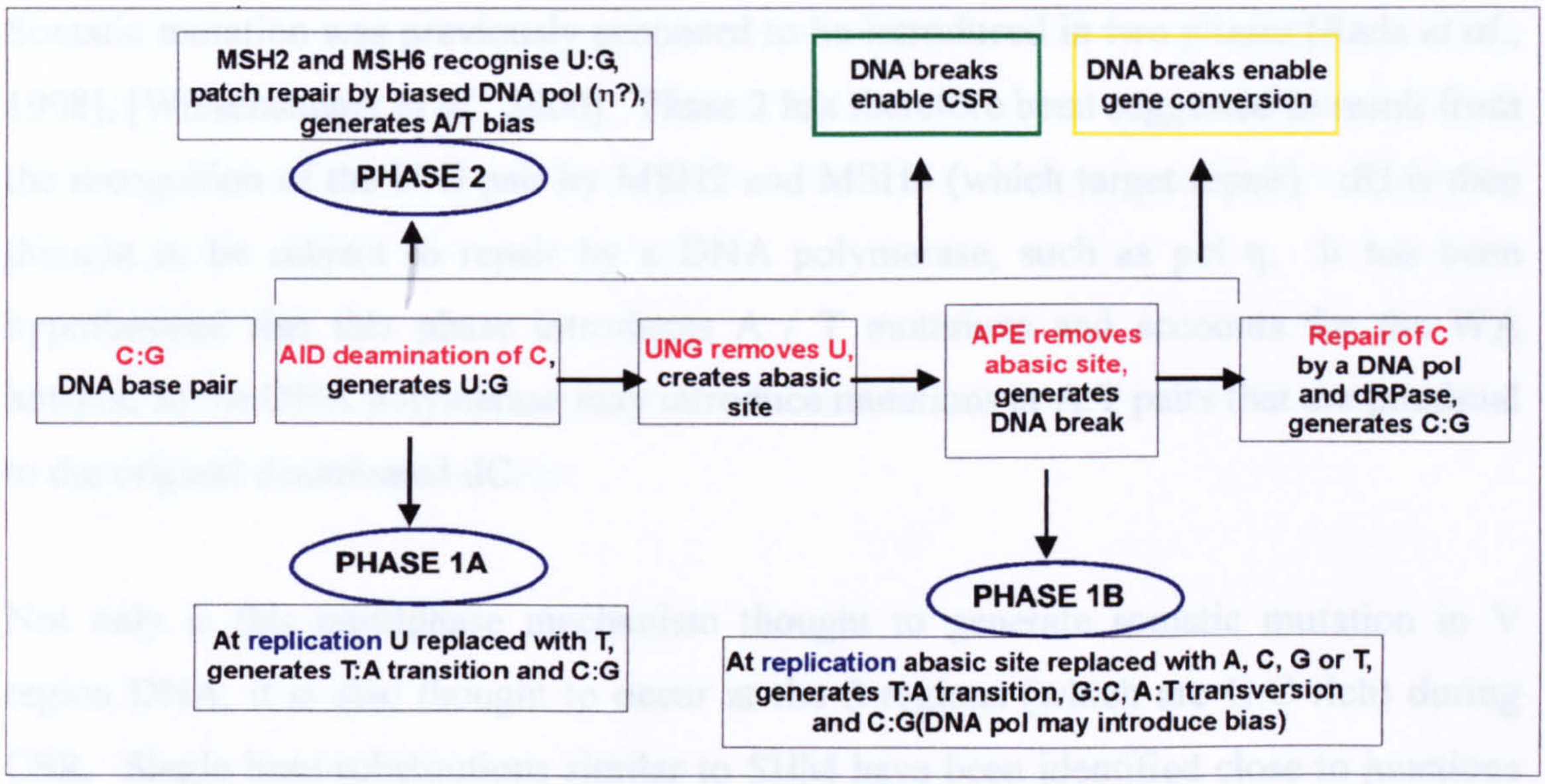


Fig 1.9.1; The proposed mechanism by which deamination of cytosine by AID may lead to SHM, CSR and gene conversion [adapted from Petersen-Mahrt et al., 2002]. The deamination of cytosine by AID leads to SHM and gene conversion in V region DNA, or CSR in C_H region DNA. Conventional base excision repairs the deaminated C correctly after the action of uracil-DNA glycosylase (UNG), apyrimidic endonuclease (APE), a DNA polymerase (pol) and deoxyribophophodiesterase (dRPase). Somatic mutation is thought to occur in two phases [Rada *et al.*, 1998], [Wiesendanger *et al.*, 2000]. In the first phase, if replication occurs across dU, transitions are generated (1a) or alternatively if replication occurs across the abasic site (1b), both transitions and transversions result. The DNA polymerase involved in phase 1b may introduce a nucleotide bias during repair. In the second phase, MSH2 and MSH6 are thought to recognise dU and direct repair by a DNA pol, η for example. During repair, this DNA pol is thought to introduce mutations at A:T. The majority of CSR event are thought to occur by APE generation of DNA breaks on opposite strands [Rada *et al.*, 2002]. Gene conversion is similarly reliant on the generation of double stranded DNA breaks.

If after AID deaminates C→U, DNA replication occurs while dU is still in place, replication of the dG strand results in the regeneration of a C:G pair. However, when the dU strand is replicated, the uracil would be replaced by thymine, resulting in mutation to T:A. This phase is termed phase 1A and would generate a C→T (and therefore, on the opposite strand, a G→A) transition [Petersen-Mahrt *et al.*, 2002]. This transition bias is consistent with that observed in SHM and may correspond to the RGYW / WRCY hotspot motif. The alternative is that DNA replication may occur after UNG activity, but before the abasic site is removed (phase 1B). In this instance, dU may be replaced with any nucleotide, with two of the three mutating alternatives resulting in a transversion.

Somatic mutation was previously proposed to be introduced in two phases [Rada *et al.*, 1998], [Wiesendanger *et al.*, 2000]. Phase 2 has therefore been suggested to result from the recognition of the U:G pair by MSH2 and MSH6 (which target repair). dU is then thought to be subject to repair by a DNA polymerase, such as pol η. It has been hypothesised that this phase introduces A / T mutations and accounts for the WA hotspot, as the DNA polymerase may introduce mutations at A:T pairs that are proximal to the original deaminated dC.

Not only is this multiphase mechanism thought to generate somatic mutation in V region DNA, it is also thought to occur at the S regions (which are G:C rich) during CSR. Single base substitutions similar to SHM have been identified close to junctions of CSR [Chen *et al.*, 2001] and are thought to result from unsuccessful attempts to switch. Successful CSR would only occur in situations where DNA breaks due to the deamination of proximal cytosines occurred on opposite strands and the necessary repair factors were present [Petersen *et al.*, 2001].

This correlates with the observation that UNG-deficient mice have an inhibited ability to switch, as UNG would be needed for the DNA break to be introduced by the apyrimidic endonuclease [Rada *et al.*, 2002], although this hypothesis may be complicated by reports that MSH2 and MSH6 are also important in CSR [Ehrenstein *et al.*, 1999], [Schrader *et al.*, 1999], [Schrader *et al.*, 2002]. In a similar fashion, gene conversion is also thought to be mediated by AID when DNA double strand breaks are generated and the appropriate repair enzymes present [Petersen-Mahrt *et al.*, 2002].

How SHM and CSR are targeted to the appropriate DNA is not currently understood any further as a result of the identification of AID. While AID appears to be the only B cell specific molecule needed to induce CSR and SHM [Martin *et al.*, 2002], [Okazaki *et al.*, 2002], it is possible that a ubiquitously expressed molecule, when associated with AID, is able to generate specificity. For example, APOBEC-1 is only active when present with apobec-1 complementation factor, which has been proposed to be responsible for the efficient targeting of APOBEC's activity [Neuberger *et al.*, 2003]. It is also possible the high level of immunoglobulin gene transcription has a role in targeting AID activity [Martin and Scharff, 2001]. The cofactor(s) needed to associate with AID to enable SHM may be different to that necessary for CSR, introducing a

further level of control and possibly explaining why SHM and CSR can occur separately [Oppezzo *et al.*, 2003], [Ta *et al.*, 2003]. Without such targeting it is not surprising that inappropriate AID expression appears to be associated with malignancy [Okazaki *et al.*, 2003], [Greeve *et al.*, 2003].

This concludes the summary of information necessary to understand the basis of the research undertaken in this thesis.

Chapter 2

Aims.

2.1 Aims of the project.

In chapter 1, the association between IgE and allergic disease and also the main concepts in the generation of high affinity IgE were discussed. Allergen-specific IgE^+ plasma cells have been demonstrated to occur locally in the nasal mucosa of allergic rhinitis patients [KleinJan *et al.*, 2000] and local production of IgE protein in the allergic nasal mucosa has been demonstrated *ex vivo* [Smurthwaite *et al.*, 2001].

In addition, it has been demonstrated that CSR and SHM occur outside of the conventional sites of lymphoid tissue, particularly in disease states such as autoimmunity, characterised by chronic antigen stimulation (see section 1.6). As the allergic mucosa is chronically stimulated by allergen and responds by synthesis of local IgE, it has been hypothesised that a similarly localised molecular response, involving SHM, clonal expansion and CSR may also occur in the allergic mucosa.

If the allergic response was demonstrated to be either entirely, or partially a consequence of locally instigated molecular events, a wider range of therapeutic targets would be available. Localised therapy would not only avoid systemic side-effects, for example, inhibition of CSR or SHM would only be viable at the local level, but may also prove successful with patients for whom conventional drug therapies are ineffective. Inhibition of high affinity IgE should be an ideal drug target, because of the redundancy of IgE for beneficial purposes in the developed world.

Analysis of ε germline transcripts has suggested that local CSR may occur in the allergic lung mucosa [Ying *et al.*, 2001], although while ε germline transcription preceeds successful CSR to IgE, B cells may not necessarily complete such a switch. However, further work on the allergic lung mucosa has demonstrated, by analysis of V_H region mRNA, the presence of clonally related B cells of different isotypes, supporting the suggestion of local CSR and additionally, SHM and clonal expansion [Snow *et al.*, 1999]. The lung is known to be supported by BALT, which in the mouse, is able to respond to antigen challenge by the formation of germinal centres [Chvatchko *et al.*, 1996]. However, although evidence suggests local SHM and CSR in the human allergic lung mucosa, there have been no reports of local germinal centre formation.

While the upper and lower respiratory tracts are considered by some clinicians to be one organ [Mygind and Jacobi, 1997], secondary lymphoid tissue is not present in the human nasal mucosa, the closest site being Waldeyer's ring in the pharynx, 8 – 10 cm distal from the inferior turbinate. However, local ε germline transcription also occurs in response to allergen stimulation in the allergic nasal mucosa [Durham et al., 1997], [Cameron *et al.*, 2000], although no supporting work, such as that carried out by Snow *et al.* in the lung, had been attempted. Additionally, no research has been conducted to determine whether local SHM and clonal expansion are also features of the allergic nasal mucosa.

Such local activity in the nasal mucosa may be a consequence of germinal centre-like activity in non-lymphoid tissue. Immunohistochemical analysis has suggested that the allergic nasal mucosa is particularly rich in both immune cells, including B lymphocytes, T lymphocytes and dentritic cells [Fokkens *et al.*, 1990] and consequentially with cytokines, such as IL-4. The local allergic mucosa may therefore be capable of acting as a specialised microenvironment, in which chronic allergen challenge results in the activation of local B cells, local CSR driven by IL-4 and also local SHM and clonal expansion.

The aims of this project were therefore to analyse the allergic nasal mucosa, to determine whether there was evidence to support the hypothesis of local CSR and SHM. The main technique employed had previously been used in analysis of the allergic lung mucosa [Snow *et al.*, 1999] and enabled the successful examination of local CSR, SHM and clonal expansion in the same sample. This goal was not achievable at the initiation of this project by any other experimental means. Initially therefore, V_H region sequences were RT-PCR amplified from IgE^+ B cells from nasal biopsies taken from the inferior turbinate of allergic rhinitis patients.

If a diverse population of IgE^+ B cells were shown to be present in the allergic nasal mucosa, then it was hypothesized that the presence of closely related IgE^+ B cell clones in a small area would suggest that they had been subject to local SHM and clonal expansion. It would be much less likely that such cells would have each migrated from Waldeyer's ring, for example and yet still remained in close proximity to their sister clones. Furthermore, if related B cell clones expressing different isotypes were

isolated, applying the same principle, it would be likely that local CSR had occurred. Later in the project, immunohistochemistry and the analysis of AID mRNA expression were also introduced, in response to the development of both this project and research published by other groups.

Chapter 3

Materials and methods.

3.1 Materials.

All chemicals were supplied by Sigma (Poole, UK) unless detailed otherwise. Tissue culture plastics were supplied by Invitrogen (Paisley, UK) unless otherwise stated. Ultrapure water was obtained from a MilliQ UF Plus machine supplied by Triple Red (Thame, UK) and was used at a purity of 18.2 mΩ / cm. Reagents were prepared as follows:

3.1.1 LB culture medium.

5 g of powdered LB (Invitrogen) was dissolved in 250 ml of MilliQ water and immediately autoclaved. The appropriate antibiotic was added immediately prior to use, ampicillin at a final concentration of 100 μg / ml and kanamycin at a final concentration of 50 μg / ml.

3.1.2 LB culture plates.

8 g of powdered LB agar (Invitrogen) was dissolved in 250 ml of MilliQ water and immediately autoclaved. The LB agar was cooled to 50°C before the appropriate antibiotic was added (concentrations as above), mixed and the LB agar poured onto eight petri dishes.

3.1.3 Xylene cyanol loading buffer.

In order to prevent the V_H-Cε PCR products from being obscured by the conventional bromophenol blue loading dye after being subjected to agarose gel electrophoresis and visualised under UV light, a loading buffer constituting 25 mg of xylene cyanol and 4 g of sucrose dissolved in 9.5 ml of MilliQ H_2O was used in all samples.

3.1.4 TAE electrophoresis buffer.

TAE electrophoresis buffer was used to make agarose gels and also used as electrophoresis running buffer (section 3.2.26). A 50X TAE stock was made and diluted as necessary, 242 g of Tris base was added to 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0). The volume was then made up to 1 litre with MilliQ H_20 and the pH adjusted to 7.2, 25μl of ethidium bromide was added per litre of 1X TAE buffer.

3.1.5 TBS wash buffer.

TBS was used as a wash buffer in immunohistochemistry experiments (sections 3.2.38 - 41). One sachet of TBS powder (Dako, Ely, UK) was dissolved in 1 litre of MilliQ water to make a pH 7.6 solution and then filter sterilised.

3.1.6 Denaturing buffer.

Denaturing buffer for southern blot analysis was made by dissolving 315.5 g of NaCl and 28.8 g of NaOH in a final volume of 1800 ml of MilliQ water. This gave a final concentration of 3 M NaCl and 0.4 M NaOH.

3.1.7 Transfer buffer.

Transfer buffer for southern blot analysis was made by dissolving 315.5 g of NaCl and 0.6 g of NaOH in a final volume of 1800 ml of MilliQ water. This gave a final concentration of 3 M NaCl and 0.01 M NaOH.

3.1.8 SSC wash buffer.

SSC buffer for southern blot analysis was made to a 20X stock by dissolving 156.6 g NaCl and 79.6 g sodium citrate in a final volume of 900 ml of MilliQ water. This stock solution was then further diluted with water as necessary. This gave a final concentration of 3 M NaCl and 0.3 M sodium citrate.

3.1.9 Yssels media.

Yssels media was made according to that previously detailed [Yssel *et al.*, 1984]: 50 ml fetal calf serum (Invitrogen), 2mM glutamine (Invitrogen), 100 IU / ml penicillin (Invitrogen), 100µg / ml streptomycin (Invitrogen), 2×10^{-5} M ethanoalanine, 2.5µg / ml BSA, 1µg / ml palmitic acid, 1µg / ml linoleic acid and 1µg / ml oleic acid were each added to 500 ml of Iscoves media (Invitrogen). Immediately prior to use 35µg / ml transferrin and 5µg / ml insulin were added.

3.2 Methods.

3.2.1 Recruitment of human subjects for nasal biopsies.

Human subjects were recruited according to the conditions approved by the Royal Brompton Hospital's local ethics committee. Male and female donors between the ages of eighteen and fifty-five were recruited from the Royal Brompton Hospital Allergy Clinic (London, UK) or by advertisement in the local press. None of the patients had received immunotherapy. Any steroid medication was discontinued at least two weeks before and anti-histamine at least two days before nasal biopsy. While the recruitment of smokers was avoided, they were not eliminated from the cohort of patients detailed below. There was no apparent difference in results obtained from the smokers compared to the non-smokers in this study.

The allergic status of the donors was assessed by their medical history, skin-prick tests and radioallergosorbency test (RAST) to detect serum allergen specific IgE. Total serum IgE was also measured. The maximum limit of detection for serum allergen specific IgE was 101 IU / ml (normal < 0.35 IU / ml). Normal total serum IgE was taken to be within the range of 3 – 150 IU / ml. Allergic rhinitis patients selected for the PCR amplification of V_H-Cε sequences from the nasal mucosa exhibited a total serum IgE >200 IU / ml wherever possible, to ensure successful PCR amplification, as preliminary work suggested that while the serum IgE would not correlate exactly with local IgE, V_H-Cε PCR products were harder or impossible to amplify from patients exhibiting lower serum IgE values. Even when patients exhibiting a total serum IgE >200 IU / ml were recruited, V_H-Cε PCR products could not always be amplified from every sample of the nasal mucosa, presumably as a result of variation between the random composition of different samples.

3.2.2 Clinical data from allergic, normal and non-atopic subjects.

The clinical details of subjects from whom data is presented in this study are listed below in Table 3.2.2.1, although when of particular importance they are again detailed in the main text. Patients from whom nasal biopsies were taken for immunohistochemical analysis are detailed in the text only. Normal total serum IgE is considered to be 3 – 150 IU / ml and normal specific IgE <0.35 IU / ml. Patients were classified as in-season on the basis of the grass pollen season, encompassing June, July and August.

Patient	Age	Sex	Status at biopsy [a)]	Total serum IgE (IU / ml)	Specific serum IgE (IU / ml) [b)]	Allergies [c)]
SL5	27	F	I / P	164	HDM = 1.2 G = 43.5 T = 1.51 M = 3.44	HDM, G, T, B, C, AF, A
CD6	19	M	I / P	990	HDM = 1.28 G = 72.9 T = 101	HDM, G, M, T, B, C, D
JB7	37	M	O / P	236	G = 9.25 C = 11.1	HDM, G, C, D, CL
CM10	18	F	O / P	382	HDM = 1.09 G = 101 C = 3.36	HDM, G, M, T, B, C, D, H, CL
HD14	21	F	I / P	787	HDM = 2.68 G = 101	HDM, G
SO16	37	M	I / P	514	G = 101	G, T, B, D
HD17	22	F	I	2745	G = 101 C = 0.39	G
AP19	38	F	I / P	414	HDM = 1.23 G = 60.2 C = 32.1	HDM, G, C, D, H, CL
DR20	31	M	I / P	892	HDM = 101 G = 97.9 C = 1.41	ND
TL22	38	F	N	ND	ND	None
JH23	25	M	P	357	HDM = 1.1 G = 42.3	G
SJ24	36	F	O / P	1264	HDM = 11.4 G = 58.5 C = 29.3	ND (severe eczema)
TL25	27	M	I / P	304	G = 13.1 C = 3.24	G, M, T, B, C, D
ZC27	20	F	I	526	G = 95.6	G

GJ29	55	M	Non-atopic	1834	AF = 0.71	None
CA30	26	F	O / P	1025	HDM = 0.36 G = 101	G, D
SLT1	38	F	O / P	ND	ND	HDM, G, B
JF	31	M	N	ND	ND	None
JM	27	M	N	ND	ND	None
VC	28	F	N	ND	ND	None
ST	32	M	N	ND	ND	None

Table 3.2.2.1: Clinical data from allergic, non-atopic and normal subjects from whom data is presented in this study. a) In-season (I), out-of-season (O), perennial (P), normal (N). b) Determined by RAST for; house dust mite (HDM), mixed grass (G), tree (T), cat (C). c) Determined by a skin-prick test ≥2 mm diameter as; *Dermatophagoides* (house dust mite) (HDM), *Phleum pratense* (grass pollen) (G), mugwort (M), three trees (T), silver birch (B), cat (C), dog (D), horse (H), *Aspergillus fumigatus* (AF), *Cladosporium herbarum* (CL), *Alternaria alternata* (A). ND = not determined.

3.2.3 Procedure for the taking of a nasal biopsy from the inferior turbinate for RNA extraction.

All nasal biopsies were performed at the Royal Brompton Hospital, London, UK, with the patients' written informed consent and under the approval of the Royal Brompton Ethics Committee. Biopsies were taken with Gerritsma forceps according to a previous detailed protocol [Durham *et al.*, 1992]. Briefly, a 2.5 mm^3 biopsy was randomly taken from the under surface of the inferior turbinate, behind the anterior insertion into the lateral wall of the nose and 8-10 cm distal to the nearest lymphoid tissue, Waldeyer's ring in the pharynx. The random nature of the biopsies prevented the sampling of any defined cell populations from within the inferior turbinate.

Biopsies were immediately placed in 1.5 ml chilled Hank's balanced saline solution (Invitrogen) and mixed to remove any surface blood. Each biopsy was then carefully

transferred with a pipette tip into a cryotube, snap frozen in liquid N_2 and stored at –70°C until use.

3.2.4 Tissue samples obtained from inferior turbinectomies.

Inferior turbinates were obtained as a by-product of routine surgery for turbinate sectioning, carried out by Ms. E. Chevretton at Guy's Hospital, London, UK. Tissue was obtained with the approval of the Guy's Research Ethics Committee and with the patient's written, informed consent.

3.2.5 Procedure for the taking of a nasal biopsy from the inferior turbinate for immunohistochemical analysis.

A drop of OCT embedding medium (Merck, Hoddesdon, UK) was placed on a small rectangle of thin cardboard (0.8 cm x 1.5 cm). Biopsies were taken as detailed in section 3.2.3, then placed into the OCT, such that the epithelium was perpendicular to the card. The embedded biopsy on the cardboard was then lowered horizontally into a beaker of isopentane (pre-chilled in liquid N_2). The frozen biopsy was stored at –70°C until used for immunohistochemical analysis (chapter 6).

3.2.6 In vivo allergen challenge of the nasal mucosa.

In order to challenge the allergic nasal mucosa *in vivo* with grass pollen allergen (chapter 8). Two actuations of grass pollen (520 BU /spray), (ALK, Hungerford, UK) were sprayed into the selected nostril. After 15 min another two actuations were administered. All patients reported symptoms of rhinitis at the end of the procedure. All grass pollen challenges took place outside of the pollen season. After five, six or seven days, nasal biopsies were taken according to the usual procedure.

3.2.7 In vitro culture of nasal biopsies.

For the analysis of biopsies cultured *in vitro* (chapter 8), biopsies were taken as detailed in section 3.2.3, but were not frozen. Rather, the biopsies were transported in Hank's balanced saline solution at 4°C as quickly as possible (always within 2 hrs). Each biopsy was cultured at 37°C in a round bottomed 96 well plate (Nunc, New York, USA) with pre-warmed Yssels medium, IL4 (200 IU / ml) (R&D Systems, Minneapolis, USA) and anti-CD40 (1μg / ml) (Pharmingen, San Diego, USA) for six days. The nasal biopsy was then removed, placed in a cryotube, snap frozen in liquid N_2 and stored at –

70°C until use. The cell pellet from the culture supernatant was also frozen at –70°C. All manipulation took place in a class II laminar flow hood under sterile conditions.

3.2.8 Isolation of human peripheral blood mononuclear cells from whole blood.

25 ml blood was immediately mixed with acid citrate dextrose (1ml in a final volume of 10 ml). Whole blood was diluted by mixing with 25 ml phosphate buffered saline (PBS), (Invitrogen) before peripheral blood mononuclear cells (PBMC) were extracted with Ficoll-Paque (Pharmacia, Ontario, Canada) according to the manufacturers instructions in a class II laminar flow hood under sterile conditions. Briefly, 50 ml of diluted blood was carefully layered onto Ficoll with a 3 : 4 ratio of Ficoll : blood, such that 20ml of blood was added to each of two tubes containing 15 ml Ficoll and 10 ml blood was added to one tube containing 7.5 ml of Ficoll. The tubes were then centrifuged at 2000 rpm for 20 min at room temperature in a benchtop centrifuge with the brake off. A pasteur pipette was then used to remove the white mononuclear cell layer in between the serum and the Ficoll. The PBMC were diluted 1:3 with sterile PBS and centrifuged again at 1500 rpm for 10 min at 4°C in a benchtop centrifuge with the brake on. The cell pellets were then combined and resuspended in 10 ml PBS. Approximately 4×10^7 cells were usually isolated from 25 ml whole blood.

3.2.9 Quantitation of cells.

Cells were mixed in equal volumes with 0.4% trypan blue (a viability dye that stains dead cells blue). Viable cell numbers were determined using a Neubauer Improved hemocytometer, the average number of cells within the innermost 25 large squares of the two grids, multiplied by 10 000, multiplied by the dilution factor, to generate the number of viable cells per ml.

3.2.10 In vitro culture of human PBMC.

PBMC from 75 ml whole blood were isolated as detailed in section 3.2.7 for a time-course experiment (chapter 8). 1.5×10^6 PBMC, resuspended in Yssels media were seeded into each of the inner eight wells of a 24 well flat bottomed plate (Nunc). The outer wells were filled with PBS. To each well was also added IL4 (200 IU / ml) and anti-CD40 (1μg / ml). 9×10^6 PBMC were frozen at –70°C as a day 0 control, but the PBMC cultured for the time-course of the experiment (either seven or fourteen days).

At each appropriate time point, the contents of four wells were pipetted to resuspend adhered cells, the supernatants pooled and then pulsed at high speed in a microfuge. The supernatant was discarded and the wells washed with 100 µl of PBS, which was then added to the cell pellet and pulsed again. The supernatant was again discarded and the cell pellet stored at –70°C.

3.2.11 In vitro culture of the AF10 cell line.

The IgE expressing AF10 B cell line (U266) was cultured at 37°C in sterile conditions from frozen stocks obtained from Professor Andrew Saxon, UCLA School of Medicine, USA. Between 5 x 10^5 and 5 x 10^6 cells / ml were maintained in RPMI 1640 media (Invitrogen) with 10% fetal calf serum (Invitrogen), 2 mM glutamine (Invitrogen) and 10 U penicillin / streptomycin (Invitrogen).

3.2.12 In vitro culture of the RAMOS cell line.

The AID expressing RAMOS IgM B cell line was cultured in sterile conditions from frozen stocks obtained from the ATCC (Manassas, USA). Between 2 x 10^5 and 2 x 10^6 cells / ml were maintained in RPMI 1640 media with 10% fetal calf serum, 2 mM glutamine and 10 U penicillin / streptomycin.

3.2.13 Total RNA extraction from nasal biopsies.

Total RNA was extracted from nasal biopsies with RNAWIZ buffer (Ambion, Huntington, UK) using a modified version of the manufacturers protocol: the biopsy was transferred with a pipette tip into an eppendorf tube and 250 µl of RNAWIZ added. The biopsy was disrupted with 300 vigorous pulses with a manual pellet pestle homogeniser (Anachem, Luton, UK). The homogenate was incubated at room temperature for 5 min before the addition of 50 µl of chloroform. The homogenate was shaken vigorously for 20 seconds and then incubated at room temperature for 10 min. The homogenate was centrifuged at 13 000 rpm in a benchtop centrifuge at 4°C for 15 min and then upper aqueous phase removed and placed in a new eppendorf tube. 125 µl of RNase free H_20 was added to the aqueous phase, mixed and then 250 µl of isopropanol added and again mixed well. This was allowed to incubate at room temperature for 10 min before being centrifuged at 13 000 rpm at 4°C for 15 min. The supernatant was removed and the pellet washed with 250 µl of cold 75% ethanol and then centrifuged at 13 000 rpm at 4°C for 5 min. The supernatant was again discarded

and the pellet air-dried for 10 min before being resuspended in 20 µl RNase free H_20. All tubes were RNase free and RNase free filtered pipette tips were used at all times when preparing and handling RNA. RNA samples were stored at –70°C.

3.2.14 Total RNA extraction from PBMC.

1 ml of RNAWIZ buffer was used to resuspend the PBMC cell pellet isolated from 25 ml of whole blood (approximately 4 x 10^7 cells). This homogenate was divided into four aliquots each of 250 µl and then the protocol followed as detailed in section 3.2.12 to isolate total RNA.

3.2.15 Total RNA extraction from cell lines.

The cells were shaken gently, then 2 x 10^6 cells harvested and centrifuged in a Sorvall RT 6000 D centrifuge at 1500 for 7 min at room temperature. The supernatant was removed and the pellets frozen at –70°C for at least 30 min to aid homogenisation. The cell pellet was resuspended in 500 µl RNAWIZ buffer and then the protocol detailed in 3.2.12 followed in order to isolate total RNA, except double volumes of all reagents were used.

3.2.16 Quantitation of RNA and DNA.

Quantitation of total RNA and DNA was carried out by spectophotometry at 260nm on a Cary UV spectrophotometer. Only OD readings between 0.1 and 1 were viewed as reliable. For quantitation of RNA, 2 µl was added to 98 µl of H_2O and the OD reading multiplied by dilution (50), multiplied by the concentration of RNA that absorbed 1 OD unit at 260 nm (40 µg / µl) to determine actual concentration of RNA in the sample (µg / µl). For DNA (generated by minipreparation of plasmids, for example) 10 µl was added to 90 µl of H_20 and the OD reading multiplied by the dilution factor of 10 and then multiplied by the concentration of DNA that absorbed 1 OD unit at 260 nm (50 µg / µl) to determine the actual concentration of DNA in the sample (µg / µl).

3.2.17 Synthesis of cDNA.

Initially in the study, an oligo dT primer was used to make cDNA. This protocol was mainly applied to the samples analysed in chapters 4 and 5 (with the exception of the subject GJ29): 5 µg of total RNA was denatured at 70°C for 2 min and then placed on ice, before adding 5X first strand buffer (Invitrogen), DTT to a final concentration of

5mM (Invitrogen), dNTPs to a final concentration of 0.4 mM each (Invitrogen), 0.2 μg oligo dT primer, 16 U RNase OUT (Invitrogen), 400 U M-MLV reverse transcriptase (Invitrogen) and the appropriate quantity of RNase free H_20 to a 40 μl reaction. The reaction was then incubated at 42°C for 1 hr.

A modification to this protocol was suggested by a colleague and was used to make cDNA from GJ29 and later samples. This generated a greater quantity of cDNA that appeared to be of better quality: 5 μg of total RNA was denatured at 70°C for 2 min and then placed on ice, before adding 5X first strand buffer, DTT to a final concentration of 5mM, dNTPs to a final concentration of 0.4 mM each, 0.2 μg oligo dT primer, 5 μg $d(N)_{10}$ random primers (Molecular Biology Unit, KCL, London, UK), 16 U RNase OUT, 400 U M-MLV reverse transcriptase and the appropriate quantity of RNase free H_20 to a 40 μl reaction. This was then incubated at 37°C for 10 min, 42°C for 40 min and then 50°C for 10 min. 160 μl of sterile H_20 was then added to the cDNA before incubating at 100°C for 2 min.

3.2.18 PCR amplification of V_H-Cε cDNA transcripts.

All PCR primers were manufactured by the Molecular Biology Unit, King's College London.

The V_H-Cε PCR was designed such that transcripts encoding both secreted and non-secreted forms of IgE were amplified. Germline transcripts and unrearranged genes were not amplified by this PCR. Initially, in order to PCR amplify V_H-Cε transcripts (approx. 450 bp) *Taq* DNA polymerase was used as follows; 5 μl of cDNA was added to a 50 μl PCR reaction that included family-specific primers homologous with the V_H region leader sequence (V_H1L-V_H6L, V_H7 being amplified by VH1L) at 0.2 μM and a Cε1 specific primer (Cε1) at 0.2 μM. 10X buffer, dNTP's each at 0.5 mM, 1.5 mM $MgCl_2$ and 2.5 U of Platinum *Taq* DNA polymerase (Invitrogen) were also included. The reaction was initially denatured at 95°C for 5 min, then subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min before a final extension at 72°C for 5 min. All PCRs were carried out in a Perkin Elmer GeneAmp 2400 PCR machine.

5 μl of the first PCR reaction was transferred into a second, nested, PCR that differed only in that it included family-specific primers homologous with FWR1 of the V_H region (V_H1F-V_H6F, V_H7 being amplified by V_H1F) and an inner Cε1 primer (Cε2). This PCR had an initial denaturation at 95°C for 5 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 5 min.

After the analysis of data from patient SL5 (chapter 4), all subsequent V_H-C_H amplification utilised the proof-reading *Pfu* DNA polymerase as follows; 5 μl of cDNA was added to a 50 μl PCR reaction that included family-specific primers homologous with the V_H region leader sequence (V_H1L-V_H6L, V_H7 being amplified by V_H1L) at 0.5 μM and a Cε1 specific primer (Cε1) at 0.5 μM. 10X buffer, dNTP's each at 0.25 mM and 1.26 U of *Pfu* DNA polymerase (Promega, Southampton, UK) were also included. The reaction was initially denatured at 95°C for 2 min, then subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min, and extension at 72°C for 2 min before a final extension at 72°C for 10 min.

5 μl of the first PCR reaction was transferred into a second, nested, PCR that differed only in that it included family-specific primers homologous with FWR1 of the V_H region (V_H1F-V_H6F, V_H7 being amplified by V_H1F) and an inner Cε1 primer (Cε2). This PCR had an initial denaturation at 95°C for 2 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 2 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min.

When a particular V_H gene class was analysed just the appropriate V_H region primers were used.

The primers used were as follows:

V_H1L: 5'-CCATGGACTGGACCTGGA-3';

V_H2L: 5'-CAGATGGACATACTTTGTTCCAC-3';

V_H3L: 5'-CCATGGAGTTTGGGCTGAGC-3';

V_H4L: 5'-CGATGAAACACCTGTGGTTCTT-3';

V_H5L: 5'-ATGGGGTCAACCGCCATCCT-3';

V_H6L: 5'-GATGTCTGTCTCCTTCCTCAT-3';

V_H1F: 5'-CAGGTGCAGCTGGTGCAGTCTG-3';
V_H2F: 5'-GTCTTGTCCCAGGTCAACTTAAGGGAGTCTT-3';
V_H3F: 5'-GAGGTGCAGCTGGTGGAGTCTG-3';
V_H4F: 5'-CAGGTGCAGCTGCAGGAGTCGG-3';
V_H5F: 5'-GAGGTGCAGCTGCTGCAGTCTG-3';
V_H6F: 5'-CTGTCACAGGTACAGCTGCAGCAGTCAG-3';
These primers were based on V_H region primers published previously [Campbell *et al.*, 1992], [Hawkins *et al.*, 1994].

Cε1: 5'-TGTCCCGTTGAGGGAGCCTGT-3';
Cε2: 5'-GGGTCGACAGTCACGGAGGTGGCATT-3',
These primers were based on Cε primers published previously [Van der Stoep *et al.*, 1993].

3.2.19 PCR amplification of V_H-Cμ, V_H-Cα or V_H-Cγ cDNA transcripts (PCR 1 and 2).

In order to amplify V_H-Cμ, V_H-Cα or V_H-Cγ transcripts (approx. 450 bp) a protocol based on that used by previous researchers was used [Efremov *et al.*, 1993], [Snow *et al.*, 1998] (see chapter 5, particularly *Fig.* 5.2.1). This enabled the selective amplification of cDNA from any clones that were related to an IgE B cell clone, but which expressed a different antibody isotype. The conditions employed in the first PCR and the second, nested PCR were the same as detailed for V_H-Cε in 3.2.18 with the following exceptions: Only the appropriate V_H region primers were used (*e.g.* if related clones from a V_H5 family were being investigated, the V_H5 primers were used). These were used in conjunction with each of the sets of C_H primers (Cμ1, Cα1 or Cγ1 in PCR 1, Cμ2, Cα2 or Cγ2 in PCR 2) as separate reactions. If the V_H5 gene class was being amplified the primer V_H5Fm was used in PCR 2 instead of VH5F. The annealing temperature used in PCR 2 was 54°C for 2 min.

The primers used were as follows:
VH5Fm: 5'-TGCAGCTGCTGCAGTCTG-3'
Cμ1: 5'-GCTCGTATCCGACGGGGAAT-3'

Cμ2: 5'-CGAGGGGGAAAAGGGT-3'

Cα1: 5'-GGGACCACGTTCCCATCT-3'

Cα2: 5'-CTCAGCGGGAAGACC-3'

Cγ1: 5'-CGGTTCGGGGAAGTAGTCCTT-3'

Cγ2: 5'-CAGGGGGAAGACCGAT-3'

The Cμ2 and Cα2 primers were based on that published [Sahota *et al.*, 1996], [Snow *et al.*, 1997].

The PCR products from PCR 2 were subjected to agarose gel electrophoresis on a 1% agarose gel, the PCR products excised and the DNA extracted from the gel.

3.2.20 PCR amplification of signature region cDNA transcripts joined to Cμ, Cα or Cγ (PCR 3).

5μl of the gel extracted PCR 2 product (section 3.2.18) was carried forward into a third, semi-nested PCR for each isotype. This reaction utilized Cμ2, Cα2 or Cγ2 in conjunction with a primer homologous to the V_H-D region specific to the signature region of the B cell clonal family that was being investigated. The other reagents used in the PCR remained the same as that detailed in section 3.2.18.

In order to amplify the signature region specific to the family of interest in conjunction with the C_H region, PCR 3 was employed. PCR 3 used the same reagents as detailed in section 3.2.18, but the appropriate inner C_H primer was used in conjunction with a primer homologous to the V_H-D region of the particular B cell clonal family. Therefore in order to identify clones from other isotypes that may have been related to the family of V_H3 IgE B cell clones from SO16 (chapter 5), PCR 1 and 2 amplified V_H3-Cμ, V_H3-Cα and V_H3-Cγ transcripts in separate reactions. In PCR 3 a sample of the PCR 2 V_H3-Cα products was further amplified using the family specific V_H-D region primer B16V3 in conjunction with the inner IgA primer (Cα2). PCR 3 used an initial denaturation at 95°C for 2 min, then 20 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 2 min, extension at 72°C for 2 min and then a final extension at 72°C for 15 min.

B16V3: 5'-GTGAAAGCCCGTTATAGTGC-3'

In order to identify clones from other isotypes that may have been related to the family of V_H5 IgE B cell clones isolated from SO16 (chapter 5), PCR 1 and 2 amplified V_H5-Cμ, V_H5-Cα and V_H5-Cγ transcripts in separate reactions. In PCR 3 samples of the PCR 2 V_H5-Cα and V_H5-Cγ products were amplified further using the family specific V_H-D region primer (B16V5) in conjunction with the inner IgA (Cα2) and the inner IgG (Cγ2) primer in separate reactions, to generate 95 bp and 94 bp PCR products respectively. This PCR 3 used the same cycling conditions detailed above for B16V3.

B16V5: 5'-AGACATAAGAGTGGCTCG-3'

In order to identify clones from other isotypes that may have been related to the family of V_H5 IgE B cell clones isolated from AP19 (chapter 5), PCR 1 and 2 amplified V_H5-Cμ, V_H5-Cα and V_H5-Cγ transcripts in separate reactions. In PCR 3 a sample of the PCR 2 V_H5-Cμ and V_H5-Cα products were amplified further using the family specific V_H-D region primer (B19V5) in conjunction with the inner IgM (Cμ2), IgA (Cα2) and the inner IgG (Cγ2) primer in separate reactions. This PCR 3 also used the same cycling conditions detailed above for B16V3.

B19V5: 5'-TATTACTGTGCGGCGGC -3'

In order to identify clones from other isotypes that may have been related to the family of V_H3 IgE B cell clones isolated from CM10 (chapter 5), PCR1 and 2 amplified V_H3-Cμ, V_H3-Cα and V_H3-Cγ transcripts in separate reactions. In PCR 3 a sample of the PCR 2 V_H3-Cα and V_H3-Cγ products were amplified further using the family specific V_H-D region primer (B10V3) in conjunction with the inner IgA (Cα2) and the inner IgG (Cγ2) primer in separate reactions. This PCR 3 used an initial denaturation at 95°C for 2 min, then 20 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 2 min, extension at 72°C for 2 min and then a final extension at 72°C for 15 min.

B10V3: 5'-TATTACTGTGC/TGAGCCACGAA -3'

In order to identify any IgE clones in SJ24B that were related to the family of IgE^+ V_H4 B cell clones isolated from the other half of the biopsy SJ24A (chapter 6), in PCR 1 and

2 V_H4-Cε transcripts were specifically amplified. In PCR 3 a sample of the PCR 2 V_H4-Cε products was amplified further using the family specific V_H-D region primer (SJ24B3) in conjunction with the inner IgE (Cε2) primer. This PCR 3 used an initial denaturation at 95°C for 2 min, then 20 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 2 min, extension at 72°C for 2 min and then a final extension at 72°C for 15 min and generated PCR products of 175 bp.

SJ24B3: 5'-CCAGAGATTTGGGGCGTTATTACTATGGTTCAGGGACG -3'

The successful PCR products from the PCR 3 experiments were subjected to agarose gel electrophoresis on a 1% or 2% gel, the DNA extracted from the gel, cloned and sequenced.

3.2.21 PCR amplification of V_H-D-J_H IgA cDNA transcripts (PCR 4).

In order to analyse the V_H region sequence from any clones identified in PCR 2 from which the correct signature region sequence had been amplified in PCR 3 (in this study only the V_H5 IgA clones amplified from the nasal mucosa of SO16), 5 µl of the gel extracted PCR 2 products was carried forward into a semi-nested PCR 4. This used identical reagents to those detailed in section 3.2.18, except that the inner V_H5 primer (VH5Fm) was used in conjunction with a primer homologous to the D-J junction unique to the SO16 V_H5 family of clones (SO16A4). An initial denaturation at 95°C for 2 min was followed by 20 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 2 min, extension at 72°C for 2 min, before a final extension at 72°C for 15 min. Resultant PCR products (327 bp) were subjected to agarose gel electrophoresis on a 1% gel, the DNA extracted from the gel, cloned and sequenced.

SO16A4: 5'-TGGCCCCAGTAGTCAGC-3'

3.2.22 PCR amplification of CDR1-D-J_H IgA cDNA transcripts (PCR 5).

A further experiment to re-amplify the complete sequence of one of the IgA clonal family members (V_H5-IgA C1) from the CDR1 in the V_H region to Cα was carried out by PCR 5 (previously PCR 3 and PCR 4 generated either part of the full sequence). PCR 5 was again semi-nested, using 5 µl of the gel extracted PCR 2 products. The reagents were the same as detailed in 3.2.18, except that a primer homologous to the

mutated CDR1 region of the clone (B16V5C1) was used in conjunction with the inner IgA (Cα2) primer. The PCR was initially denatured at 95°C for 2 min and then was subjected to 20 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 2 min, extension at 72°C for 2 min, before a final extension at 72°C for 15 min. Resultant PCR products (308 bp) were subjected to agarose gel electrophoresis on a 1% gel, the DNA extracted, cloned and sequenced.

B16V5C1: 5'-TATAAGTTTGCCACCTATGCC-3'

3.2.23 PCR amplification of GAPDH.

The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified to confirm either that the cDNA was of sufficient quality for PCR analysis, or that approximately comparable amounts of cDNA were used in the PCR amplification of different samples. GAPDH amplification by Taq DNA polymerase was as follows; 2.5 µl of cDNA was added to a 25 µl PCR reaction that included the primers GAPDHF2 and GAPDHR2 at 1 µM, 10X buffer, 1.5 mM $MgCl_2$, dNTP's each at 0.2 mM and 1.25 U of Platinum *Taq* DNA polymerase. The reaction was initially denatured at 95°C for 5 min, then subjected to denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1 min before a final extension at 72°C for 5 min.

GAPDH amplification by *Pfu* DNA polymerase was as follows; 2.5 µl of cDNA was added to a 25 µl PCR reaction that included the primers GAPDHF2 and GAPDHR2 at 1 µM, 10X buffer, dNTP's each at 0.25 mM and 0.63 U of *Pfu* DNA polymerase. The reaction was initially denatured at 95°C for 2 min, then subjected to denaturation at 94°C for 1 min, annealing at 59°C for 2 min, and extension at 72°C for 2 min before a final extension at 72°C for 10 min.

Normally 30 cycles of PCR were undertaken, but when samples were being normalised to GAPDH expression levels, PCR products were removed at 15, 20, 25 and 30 cycles for comparison. 393 bp PCR products resulted. The GAPDH primers were designed by David Fear in the Gould group.

GAPDHF2: 5'-ATTTGGTCGTATTGGGCGCCTGGTC-3'

GAPDHR2: 5'-TCATACTTCTCATTGTTCACACCCATG-3'

3.2.24 PCR amplification of AID.

In this study, amplification of AID cDNA transcripts was carried out using both a single and a nested PCR protocol (chapter 8). To amplify AID in one PCR the following was carried out; 5 µl of cDNA was added to a 25 µl PCR reaction that included the primers AID3 and AID4 at 0.2 µM, 10X buffer, 1 mM $MgCl_2$, dNTP's each at 0.2 mM and 1.25 U of Platinum *Taq* DNA polymerase. The reaction was initially denatured at 95°C for 5 min, then 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 2 min before a final extension at 72°C for 5 min to generate a 647 bp PCR product.

When amplification of AID by a nested PCR was carried out, the initial PCR was exactly as detailed above, although the reaction was only subjected to 30 cycles. 5 µl of the initial PCR was carried over and included in the second, nested 25 µl PCR with the same reagents as detailed above except that the inner primers AID1 and AID2 at 0.2 µM were used. The reaction was initially denatured at 95°C for 5 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min before a final extension at 72°C for 5 min, to generate a 335 bp PCR product.

The primers used were as follows;

AID1: 5'-TAGACCCTGGCCGCTGCTACC-3'

AID2: 5'-CAAAAGGATGCGCCGAAGCTGTCTGGAG-3'

AID3: 5'-GAGGCAAGAAGACACTCTGG-3'

AID4: 5'-GTGACATTCCTGGAAGTTGC-3'

These primers were based on AID primers published previously [Muto *et al.*, 2000], [Revy *et al.*, 2000].

3.2.25 Southern blot analysis of AID mRNA expression.

The presence of AID PCR products was confirmed by Southern blot analysis (chapter 8). 10 µl of the nested AID PCR was subjected to electrophoresis on a 1.5% agarose gel. The agarose gel was then incubated with 200 ml of denaturing buffer shaking, at room temperature for 45 min. Nylon membrane (Sigma) was briefly immersed in

transfer buffer and then positioned under the inverted gel (also rinsed briefly with Transfer Buffer) and placed under vacuum. Once the vacuum had sealed, transfer buffer was added to cover the gel and the DNA left to transfer to the membrane for 45 min.

The nylon membrane was rinsed briefly in MilliQ water, then dried and stored at room temperature until needed. In order to probe for AID RT-PCR products, 25 ng of an AID specific probe (spanning the 3' of exon 3 and the 5' of exon 4) was labelled by random priming, briefly the probe was denatured at 100°C for 2 min and then added to a DNA labelling bead (Amersham, Chalfont St. Giles, UK), resuspended in 35 µl of H_20. 5µl of α-dCTP^{32} was added to the mix to end-label the probe. This reaction was incubated for 1 hr at 37 °C before being transferred onto a pre-spun G25 column (Roche, Basel, Switzerland) and the labelled probe harvested by centrifugation of the column at 2000g for 4 min in a benchtop centrifuge, according to the manufacturers instructions.

The membrane was placed inside a roller bottle, containing 20 ml of pre-hybridisation buffer (Ambion) and incubated, rotating, at 42°C for 45 min before 8µl of the labelled probe was added and the membrane incubated at 42°C, rotating, overnight. The probe was then removed and 200 ml of 5X SSC buffer added to the roller bottle to wash the membrane for 20 min at 42°C, rotating. The membrane was then subjected to a further two washes with 1X SSC buffer, one wash with 0.2X SSC buffer and finally one wash with 0.1X SSC buffer. The membrane was then covered with Saran Wrap and exposed to Kodak MR-1 Biomax fast film (Sigma) overnight, at room temperature, using amplifying screens. The film was then developed using a Kodak automatic developer.

AID probe; 5'- CCGGGGTGCAAATAGCCATCATGACCTTCAAAGATTATTT TTACTGCTGGAATACTTTTG -3'

3.2.26 Agarose gel electrophoresis.

DNA was subjected to agarose gel electrophoresis as follows; agarose (0.5, 1 and 2 g for a 0.5, 1 or 2% gel respectively) was dissolved in 100 ml of TAE buffer for 3 min at 70°C in the microwave. The molten agarose was left at room temperature to cool for 10 – 15 min before adding 1µl of ethidium bromide and mixing. The molten agarose was then poured into a gel tray and the appropriate sized comb added. The agarose was left

for 30 min at room temperature to solidify. The comb was removed and the agarose gel was placed, still in the gel tray in an electrophoresis tank and immersed in TAE that also contained ethidium bromide. An appropriate DNA ladder was loaded onto the gel. 1 µl of xylene cyanol loading buffer was added for every 10 µl of DNA that was then loaded into each well. A constant voltage of 115 V was then applied. When the DNA bands were separated, after approximately 35 min for most PCR products, the gel was exposed to ultra violet light. Pictures of the DNA bands evident were taken with a Kodak Poloroid camera or the Syngene Gene Genius Bioimaging System (Cambridge, UK). Gel pictures presented in this study are almost always presented as the inverse (black bands on a pale background) because of the better resolution this provided when images were processed with the Syngene Gene Genius Bioimaging System.

3.2.27 DNA ladders.

Two different DNA ladders were used in this study. The Hpa II ladder (Molecular Biology Unit, KCL) comprised DNA fragments evident between 157 bp and 710 bp generated by the digestion of the plasmid pBS Sk by *Hpa* II (*Fig.* 3.2.25.1). 7 µl of ladder was loaded into a single well of each agarose gel.

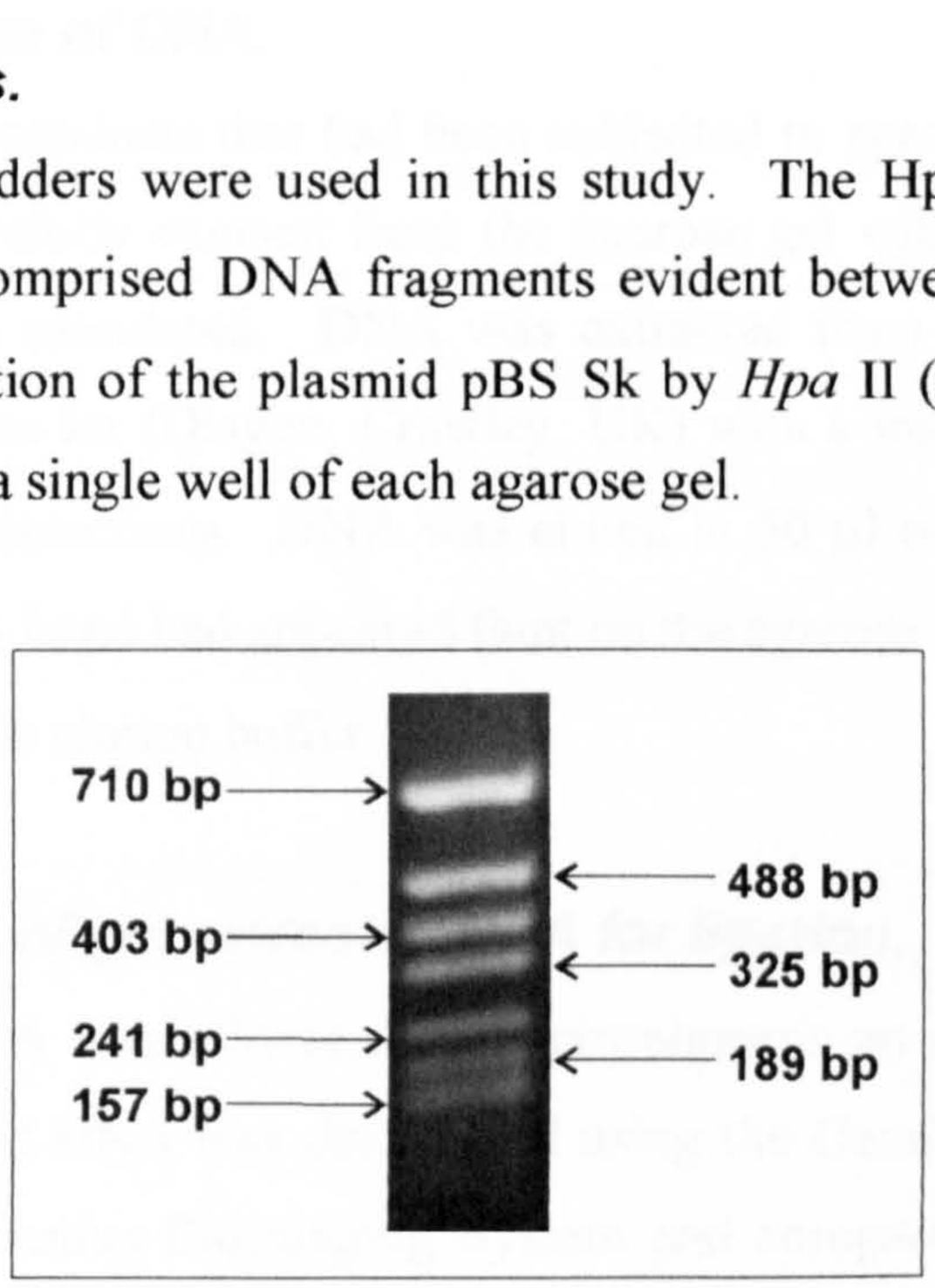


Fig. 3.2.25.1; Agarose gel electrophoresis of Hpa II ladder. 7 µl *Hpa* II ladder (MBU, King's College London) was subjected to electrophoresis on a 1% agarose gel and visualised under UV. The number of base pairs (bp) comprising each DNA fragment from 157 – 710 bp are labelled.

A 100 bp ladder (Promega) was also used. This ladder comprised a range of different DNA fragments between 100 bp and 1500 bp (*Fig.* 3.2.25.2). 5 µl of ladder was loaded into a single well of each agarose gel.

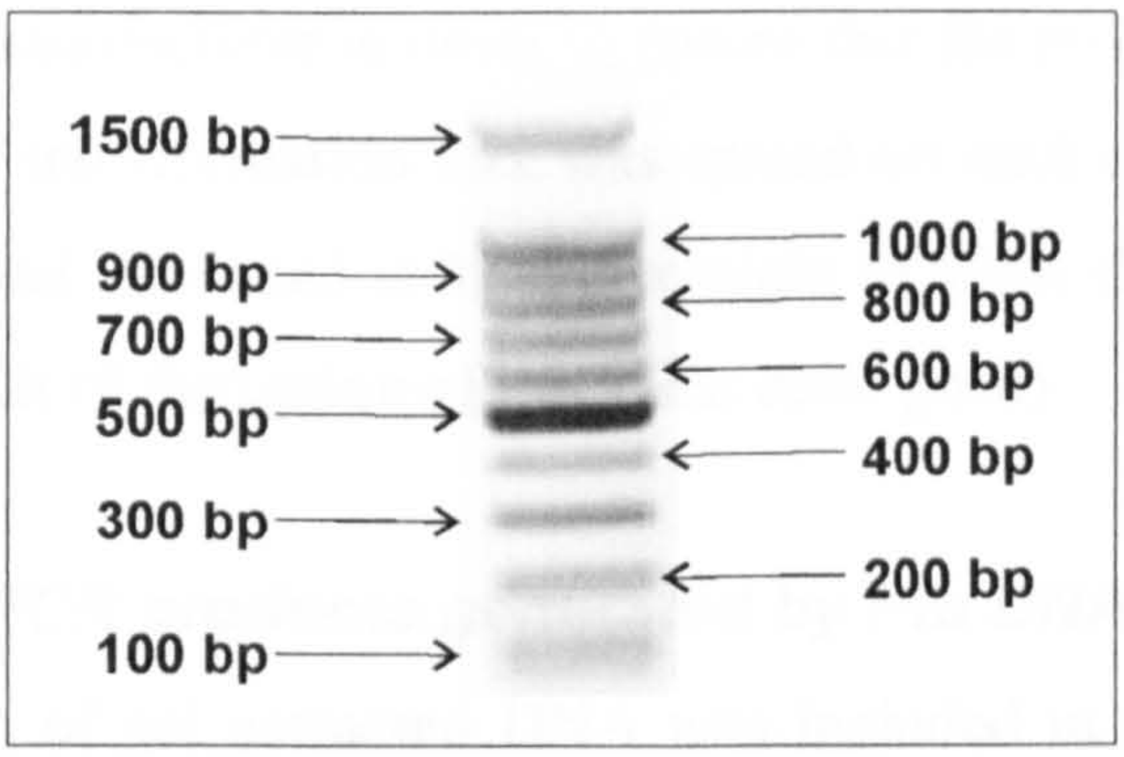


Fig. 3.2.25.2; Agarose gel electrophoresis of 100 bp ladder. 5 μl 100 bp ladder (Promega) was subjected to electrophoresis on a 1% agarose gel and visualised under UV. The number of base pairs (bp) comprising each DNA fragment from 100 –1500 bp are labelled.

3.2.28 Gel extraction of DNA.

In order to purify PCR products that had been subjected to agarose gel electrophoresis, the DNA band was carefully excised from the agarose gel with a razor blade and the weight of the gel slice calculated. DNA was extracted from the gel slice using the QIAquick Gel Extraction kit (Qiagen, Crawley, UK) with a microcentrifuge according to the manufacturers instructions. DNA was eluted in 50 μl of the elution buffer EB, unless the original DNA band had appeared faint on the agarose gel, in which case DNA was eluted in 30 μl of the elution buffer.

3.2.29 Quantitation of gel extracted DNA for ligation.

15 μl gel extracted DNA was subjected to electrophoresis on a 1% agarose gel. The intensity of the extracted DNA was determined using the GeneTools software package in the Syngene Gene Genius Bioimaging System and compared to the intensity of a known quantity of DNA from one of the DNA ladders. This enabled the approximate concentration of each DNA sample to be determined.

3.2.30 Cloning of PCR products generated by Taq DNA polymerase.

Where possible, 20 ng of gel extracted DNA was included in each ligation reaction. The 'TOPO TA cloning kit for sequencing' (Invitrogen) was used for both the ligation and transformation. The manufacturers instructions for the transformation of chemically competent *E. coli* (One Shot TOP 10 cells) was followed. Wherever relevant, the ligation and transformation was incubated for the longest amount of time

recommended by the manufacturer in order to ensure that the procedure was as efficient as possible. 100 µl of transformation mix was spread on each of three LB agar plates containing ampicillin and incubated at 37°C overnight to allow the positive selection of recombinants (as a result of disruption of the lethal *ccdB* gene).

3.2.31 Cloning of PCR products generated by Pfu DNA polymerase.

Where possible, 20 ng of gel extracted DNA was included in each ligation reaction. Where DNA from the PBMC and the nasal biopsy of the same patient was being ligated in parallel experiments, the quantity of DNA included in each was identical. The 'Zero Blunt TOPO PCR cloning kit' (Invitrogen) was used for both the ligation and transformation. The manufacturers instructions for the transformation of chemically competent *E. coli* (One Shot TOP 10 cells) was followed. Wherever relevant, the ligation and transformation was incubated for the longest amount of time recommended by the manufacturer in order to ensure that the procedure was as efficient as possible. 100 µl of transformation mix was spread on each of three LB agar plates containing kanamycin and incubated at 37°C overnight to allow the positive selection of recombinants (as a result of disruption of the lethal *ccdB* gene).

3.2.32 Overnight 3ml cultures of transformed E. coli.

3 ml overnight cultures were used to grow sufficient of a transformed bacterial colony to enable plasmid minipreparation. 3 ml of LB (with ampicillin if the DNA was generated by *Taq*, or kanamycin if generated by *Pfu*) was placed in a 50 ml universal tube. Each tube was innoculated with a single discreet transformed bacterial colony and incubated overnight, shaking (225 rpm) at 37°C.

3.2.33 Mini-preparation of plasmid DNA.

Plasmid DNA was isolated from a 3ml overnight culture. The culture was transferred to an Eppendorf tube and the *E. coli* pelleted by centrifugation in a microcentrifuge at 10,000 rpm for 5 min. Isolation of plasmid DNA from the bacteria was then carried out using the 'Wizard *Plus* SV Miniprep DNA Purification System' (Promega) by microcentrifugation, according to the manufacturers instructions. Briefly, the cell pellet was resuspended in 250 µl of Cell Resuspension Solution (Promega) and then 250 µl of Cell Lysis Solution (Promega) added. The tube was inverted four times and then 10 µl of Alkaline Protease Solution (Promega) added, again inverted four times and incubated

for 5 min at room temperature. 350 µl of Neutralisation Solution was added, inverted again four times and then centrifuged at 13,000 rpm for 13 min at room temperature in a benchtop microfuge. The lysate was then added to a Spin Column with Collection Tube (Promega) and centrifuged at 13,000 rpm in a benchtop centrifuge for 1 min at room temperature. The flowthrough was discarded and 750 µl of Wash Solution (Promega) added, centrifuged again as above and repeated with 250 µl of Wash Solution, the flowthrough discarded and then centrifuged at 13,000 rpm in a benchtop centrifuge for 2 min at room temperature. The column was transferred to a 1.5 ml Eppendorf tube and 100µl of H_20. This was centrifuged at 13,000 rpm in a benchtop centrifuge for 1 min at room temperature to elute the resuspended plasmid DNA which was stored at –20°C or below.

3.2.34 EcoRI digestion of plasmid DNA.

In order to confirm that each plasmid isolated by mini-preparation contained the expected DNA insert, 1 µl of plasmid DNA was subjected to *EcoRI* digestion in a 10 µl reaction that also included 1 µl 10X buffer, and 10 U of *EcoRI* (Promega). The restriction digest was incubated at 37°C for 2 hrs before being subjected to agarose gel electrophoresis. Any plasmid from which the expected size DNA fragment was not excised were discarded.

3.2.35 Sequencing of plasmid DNA.

Plasmid DNA containing the sequence of interest as an insert was sequenced using the M13 forward or M13 reverse primer and an ABI 3100 Automated DNA sequencer (Perkin Elmer, Beconsfield, UK) by the Advanced Biotechnology Centre (Imperial College, London, UK). Sequences of importance *e.g.* those isolated for V_H region analysis were always repeated.

M13 forward (-20): 5'-GTAAAACGACGGCCAG-3'

M13 reverse: 5'-CAGGAAACAGCTATGAC-3'

3.2.36 DNA sequence analysis.

Alignment of multiple sequences was carried out with the use of the 'GeneTool Lite' program (DoubleTwist.com). The identity of non-V_H region sequences was determined by use of the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST).

The identity of V_H region sequences was determined by use of the VBase database (www.mrc-cpe.cam.ac.uk), enabling assignment of V_H, D and J_H genes and their somatic mutations according to their closest homology to a germline sequence. When the identity of a D gene was assigned with a score of less than 50, the D gene was classified as not reliably identified (+5 being awarded for a nucleotide match, –4 for a mismatch). Where the assignment of the V_H and D gene, or the D and J_H gene overlapped, precedence was given to the V_H sequence and J_H sequence respectively, such that an apparently short D gene sequence was presented in some sequences. All the sequences were identified as having an in-frame rearrangement, implying translation of the mRNA into protein.

The signature region, defining the clonal identity of a B cell, included the V_H-D-J_H recombination junction (CDR3 / FWR4), at which non-templated (N) nucleotides were often also inserted. Genealogical trees of related B cell clones were constructed based on the premise that V_H region mutations shared by related B cell clones were acquired by SHM early in the evolution of the clonal family, while mutations unique to each B cell clone were acquired later. Mutations were detailed such that 31(2) referred to a mutation at codon 31, position 2. The polymorphic codons 31a, 31b, 52a, 52b, 52c, 82a, 82b and 82c were detailed as such. Some previous researchers had included these within the normal numbering. In this study, the polymorphic codons are distinguished as 31a, for example, rather than 32. This means that the mutation at Ala72 referred to by [Snow *et al.*, 1997] corresponds to and is detailed as Ala71 in this study.

The details of all V_H region sequences were added to a database to enable any contaminants (*i.e.* identical V_H-D-J_H sequences isolated from different patients) to be identified and eliminated from the study. Any sequences that demonstrated evidence of being a possible PCR artefact (*i.e.* the 5' of one sequence and the 3' of another) were also eliminated from the study.

3.2.37 Frozen nasal biopsy sections.

All immunohistochemistry (chapter 6) utilised 6μm sections cut at –20°C by cryostat from nasal biopsy samples mounted in OCT. Section were placed on polysine microscope slides (BDH Laboratory Supplies, Poole, UK), stored at –70 °C and used as

quickly as possible. Frozen tissue sections were used in order to preserve the integrity of the RNA for the intended RT-PCR of microdissected cells and no counter-staining was undertaken. All sections were visualised with a Zeiss Axioscop microscope (New York, USA). Images were processed using an Axiocam colour CCD camera and Axiovision software. Fluorescent images stained with FITC were visualised using a band pass filter with an excitation of 450 – 490 nm, a dichroic mirror at 510 nm and a band pass filter with an emission spectrum of 515-565 nm. Fluorescent images stained with Cy3 were visualised with a narrow pass filter with an excitation spectrum of 546 ± 12 nm, a dichroic mirror at 560 nm and a band pass filter with an emission spectrum of 575 – 640 nm. Chromogen stained sections were stored at room temperature for future reference. Fluorescent stained sections were stored in the dark at 4°C for no longer than 1 week.

3.2.38 Double staining of CD138 and IgE in nasal biopsy sections, visualised by chromogenic substrates.

The frozen nasal biopsy sections used in this protocol were fixed in 4% paraformaldehyde after being cut on the cryostat, before being snap-frozen in liquid N_2 and stored at –70°C. The sections were thawed at room temperature for 1 min before being washed twice, for 2 min in TBS. The sections were then incubated for 10 min with peroxidase blocking reagent (Dako, S2001) before again being washed for 2 min in TBS. The sections were additionally incubated in 0.2% H_2O_2 for 5 min and then washed twice for 2 min in TBS before being incubated in blocking solution for 15 min (ISCOVE's media with additionally 10% fetal calf serum, 10% normal goat serum (Vector Laboratories, Burlingame, USA) and 10% normal horse serum (Vector Laboratories), 10 U penicillin / strepamycin and 2 mM glutamine. (The antibodies were also diluted in this blocking medium). An avidin / biotin block (Vector Laboratories) was then applied, each for 10 min with a 2 min TBS wash in between.

The sections were incubated with goat anti-human IgE-biotin antibody (12.5 μg / ml), (Vector Laboratories, BA-3040) or (as an isotype control) biotinylated goat-IgG (Vector Laboratories, BI-1001) for 35 mins. The sections were washed three times for 2 min in TBS and then incubated with streptavidin-HRP (2 μg / ml), (Dako, P0397) for 35 min before washing three times for 5 min in TBS. The sections were then incubated with mouse anti-human CD138 antibody (0.1 mg / ml), (Serotec, MCA681H, Oxford, UK) or

as an isotype control, mouse MOPC21 antibody (5μg / ml), (Sigma, M7894), both for 35 min. After washing three times in TBS each for 2 min, the sections were then incubated with goat anti-mouse immunoglobulins-alkaline phosphatase antibody (20 μg / ml), (Dako, D0486) for 35 min. After washing three times in TBS for 5 min the sections were then incubated with Fuschin chromogenic substrate (Dako) and Levamisole (Dako) for 7 min, washed twice in 0.2 M sodium acetate (pH 4.6) for 5 min, then incubated with DAB chromogenic substrate (Dako) for 10 min.

The sections were finally washed in MilliQ H_20 for 5 min, before being mounted with a coverslip and Glycergel (Dako).

3.2.39 Double staining of CD138 and IgE in nasal biopsy sections, visualised by fluorescence.

The frozen nasal biopsy sections used in this protocol were fixed in 4% paraformaldehyde after being cut on the cryostat, before being snap-frozen in liquid N_2 and stored at –70°C. The sections were thawed at room temperature for 1 min before being washed twice, for 2 min in TBS. The sections were then incubated for 10 min with peroxidase blocking reagent before again being washed for 2 min in TBS. The sections were additionally incubated in 0.2% H_2O_2 for 5 min and then washed twice for 2 min in TBS before being incubated in blocking solution for 15 min (ISCOVE's media with additionally 10% fetal calf serum, 10% normal goat serum and 10% normal horse serum, 10 U penicillin / strepamycin and 2 mM glutamine. (The antibodies were also diluted in this blocking medium). An avidin / biotin block was then applied, each for 10 min with a 2 min TBS wash in between.

The sections were incubated with mouse anti-human CD138 antibody (0.1 mg / ml) or as an isotype control, mouse MOPC21 antibody (5μg / ml), for 35 minutes. The sections were washed three times for 2 min in TBS and then incubated with goat anti-mouse immunoglobulins–FITC (13.3 μg / ml), (Dako, F0479) for 35 mins, before washing three times for 5 min in TBS. The sections were then incubated with goat anti-human IgE-biotin antibody (12.5 μg / ml) or (as an isotype control) biotinylated goat-IgG (Vector Laboratories, BI-1001), for 35 min. After washing three times in TBS each for 2 min, the sections were then incubated with streptavidin-Cy3 (20 μg / ml),

(Amersham) for 35 min and then finally washed three times in TBS for 5 min before being mounted with a coverslip and fluorescence mountant (Dako).

3.2.40 Staining of CD19 in nasal biopsy sections, visualised by chromogenic substrate.

This protocol was based on that previously published [KleinJan *et al.*, 2000]. The frozen nasal biopsy sections were thawed at room temperature for 1 min before being fixed in acetone at room temperature for 10 min. The sections were then washed twice, for 2 min in TBS before being incubated in blocking solution for 15 min (ISCOVE's media with additionally 10% fetal calf serum, 10% normal goat serum and 10% normal horse serum, 10 U penicillin / strepamycin and 2 mM glutamine. (The antibodies were also diluted in this blocking medium). An avidin / biotin block was then applied, each for 10 min with a 2 min TBS wash in between.

The sections were incubated with mouse anti-human CD19 antibody (25 μg / ml), (Immunotech-Coulter, IM1283, Miami, USA) or as an isotype control, mouse MOPC21 antibody (5 μg / ml) for 60 min. The sections were washed three times for 2 min in TBS and then incubated with biotinylated goat anti-mouse IgG (10 μg / ml), (Vector Laboratories, BA-9200) for 30 min. After washing three times in TBS each for 2 min, the sections were then incubated with alkaline phosphatase-conjugated streptavidin (5 μg / ml), (Vector Laboratories, SA-5100) for 30 min. After three 5 min washes in TBS, the sections were incubated for 10 min with Fuschin chromogenic substrate and Levamisole for 10 min.

The sections were finally washed in MilliQ H_20 for 5 min, before being mounted with a coverslip and Glycergel.

3.2.41 Staining of CD19 in nasal biopsy sections, visualised by fluorescence.

The protocol described in 3.2.38 was followed until the third antibody incubation where incubation of the sections was commenced in the dark with Cy3-conjugated streptavidin (20 μg / ml), (Amersham, PA 43001) for 30 min. After three 5 min washes in TBS, the sections were then mounted with a coverslip and fluorescence mountant.

Chapter 4

Local somatic hypermutation and clonal expansion in the nasal mucosa of allergic rhinitis patients.

4.1 Introduction.

In order to determine whether somatic hypermutation and the subsequent expansion of IgE^+ B cell clones with increased affinity occurs locally within the allergic nasal mucosa, total RNA was isolated from nasal biopsy and PBMC samples from allergic rhinitis patients. cDNA from B cell V_H-Cε transcripts was amplified by PCR. V_H region somatic mutations were identified by comparison of the V_H-Cε sequences with the germline repertoire. The relatedness of the B cells was determined on the basis that sequences originating from clonally related B cells, sharing a common progenitor, exhibit identical 'signature region' sequences across the V_H-D and D-J_H junctions resulting from the unique V_H-D-J_H gene rearrangement in the progenitor. Related clones exhibited both shared and unique somatic mutations in V_H.

4.2 DNA polymerase usage for the PCR amplification of V_H-Cε.

The initial experiment to analyse V_H-Cε sequences from a nasal biopsy followed a previously published protocol [Snow *et al.*, 1997], utilising *Taq* DNA polymerase in a nested PCR reaction to amplify V_H-Cε (see 3.2.18 for details). The PCR products were subjected to agarose gel electrophoresis, extracted from the agarose gel, cloned and sequenced, according to the methods described in sections 3.2.26 - 36. This experiment generated misleading data and its results indicated the importance of using the proof-reading *Pfu* DNA polymerase in future work.

Eighteen sequences were analysed from a nasal biopsy taken from patient SL5 (see Table 4.2.1 for patient details). Of the eighteen sequences analysed, eight were different and could be divided into members of two families of clonally related IgE^+ B cells (*Fig.* 4.2.1), each family originating from a progenitor cell expressing a distinct V_H-D-J_H rearrangement that had been subject to somatic hypermutation. Identical sequences were assumed to have resulted from multiple PCR products amplified from the same cell and were represented only once in the data analysis.

Patient	Age	Sex	Status at biopsy [a)]	Total serum IgE (IU / ml) (Norm = 3 - 150)	Specific serum IgE (IU / ml) [b)] (Norm < 0.35)	Allergies [c)]
SL5	27	F	I / P	164	HDM = 1.2 G = 43.5 T = 1.51 M = 3.44	HDM, G, T, B, C, AF, A

Table 4.2.1: Clinical data from patient SL5. **a)** In-season (I), perennial (P). **b)** Determined by RAST for house dust mite (HDM), mixed grass (G), tree (T), cat (C), mixed mould (M). **c)** Determined by a skin-prick test ≥2mm as *Dermatophagoides* (house dust mite) (HDM), *Phleum pratense* (grass pollen) (G), mugwort (M), three trees (T), silver birch (B), cat (C), dog (D), horse (H), *Aspergillus fumigatus* (AF), *Cladosporium herbarum* (CL), *Alternaria alternata* (A).

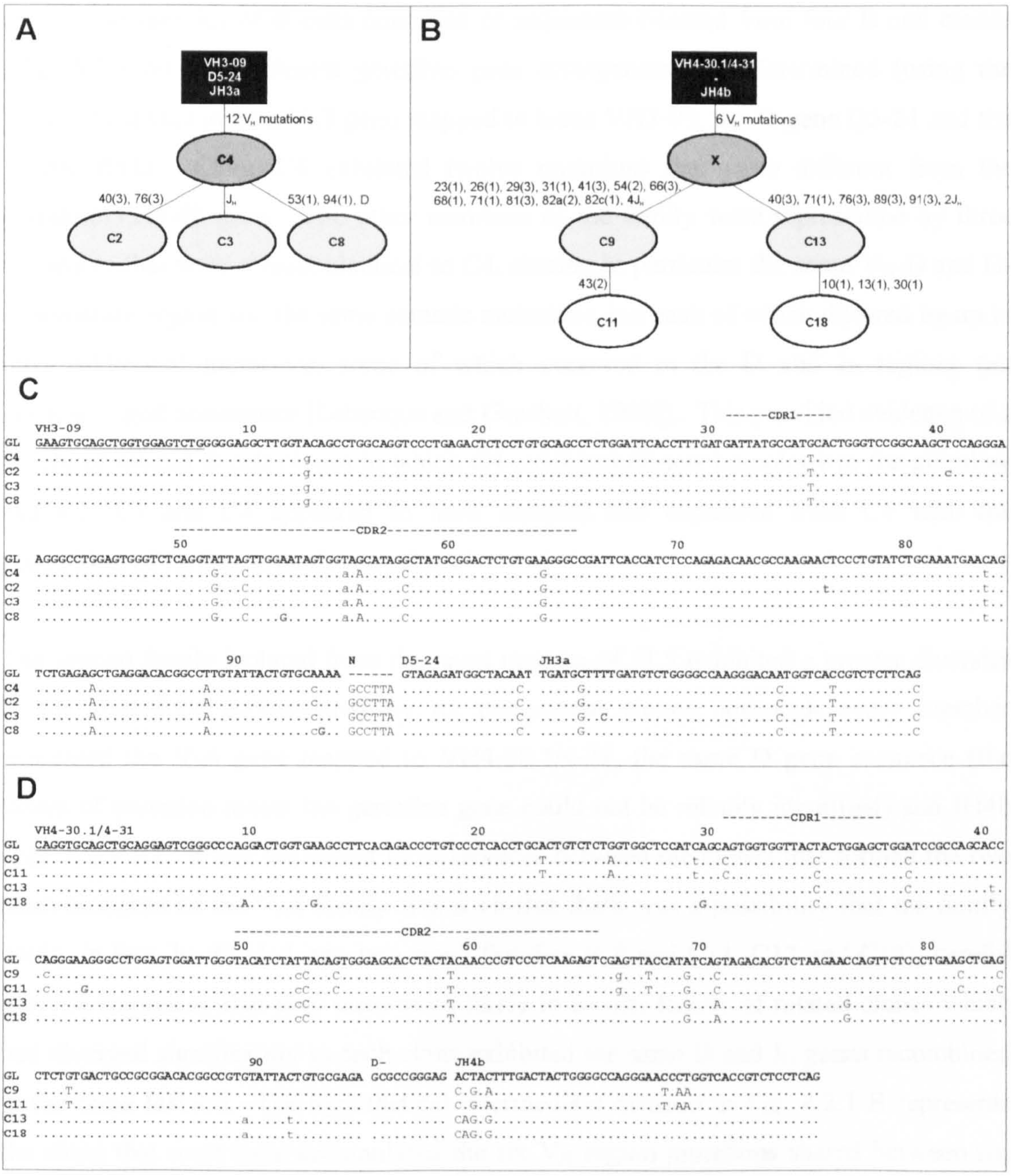


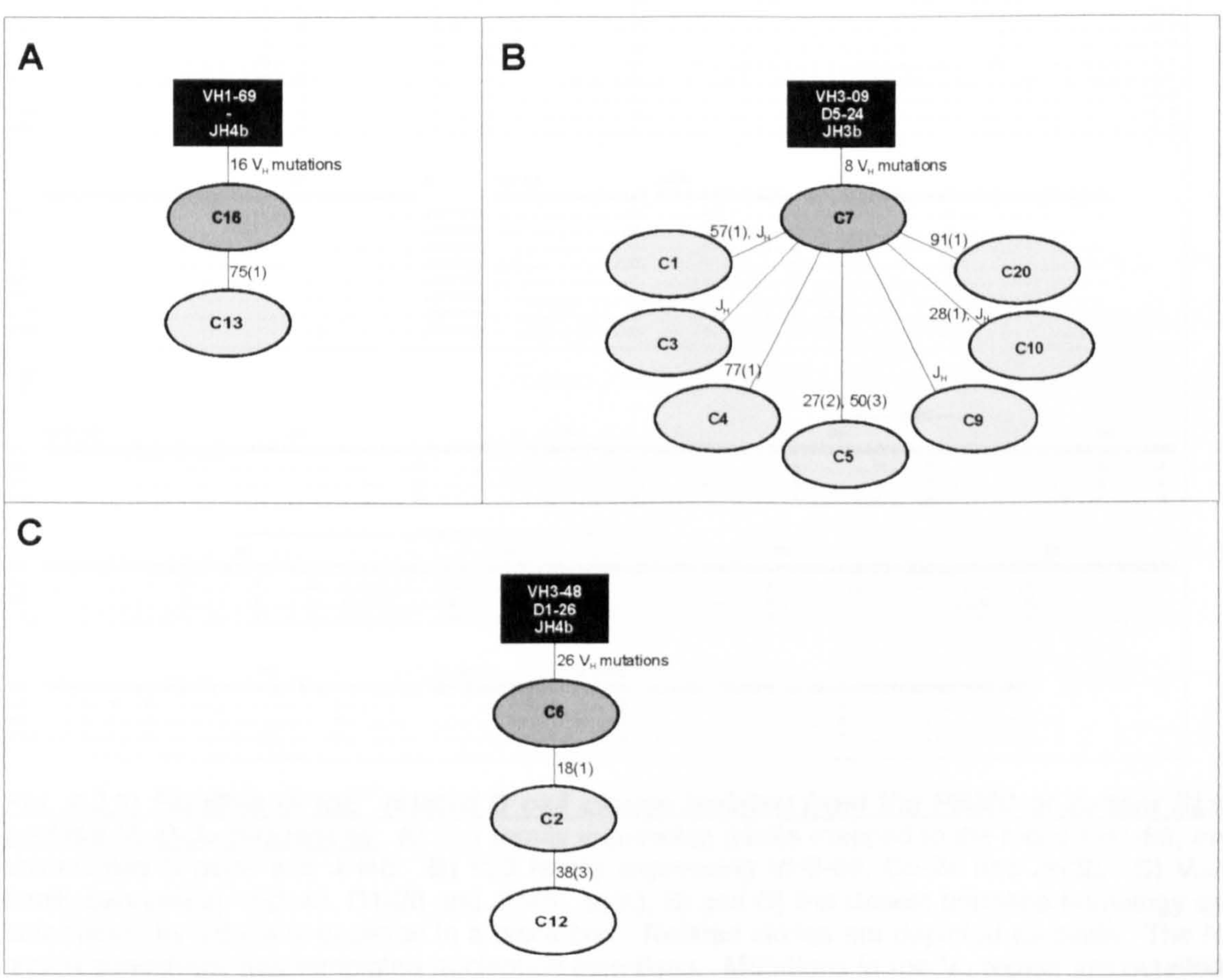
Fig. 4.2.1; Families of related IgE⁺ B cell clones isolated from the nasal mucosa of patient SL5 and the V_H-D-J_H sequences. **A)** V_H3 family expressing genes mapped to the locus VH3-09, D5-24 and JH3a. **B)** V_H4 family expressing VH4-30.1/4.31, an unidentified D gene and JH4b. In both A) and B) the closest germline homology determined by VBase is depicted in a black box. Related clones are depicted as ovals. Hypothetical clones, not isolated experimentally but which must be intermediates are denoted X. Mutations in the V_H region are detailed such that 40(3) represents a mutation at codon 40, position 3. Additional mutations in the D and J_H regions are also detailed. In **C)** and **D)** the sequences of the V_H3 and V_H4 clones are detailed respectively. The N region constitutes non-templated nucleotide insertions. GL represents the closest germline homology as determined by VBase. Primer binding regions are underlined and no mutations occurring in these regions are included. Homology to the germline is represented with a dot, whereas mutations from the germline are depicted either in upper case (if causing an amino acid replacement) or lower case (if having a silent effect upon the amino acid). Where the link between clones is dependent on one, two or three mutations, these are highlighted in red. The CDR regions are detailed.

One of the families of B cells consisted of sequences isolated from four B cell clones (*Fig.* 4.2.1 A). The closest germline gene arrangement was determined (using the VBase database) to be a V_H3 gene mapped to locus VH3-09, the D gene D5-24 and the J gene JH3a. Clone C4 exhibited twelve mutations that were different from the germline VH3-09 gene. The other members of the family were represented by three sequences that were almost identical to C4, sharing in particular the same V_H-D and D-J_H signature region and the same somatic mutations, but each of which differed by up to three additional mutations, some of which occurred in the D and J_H regions (an acknowledged occurrence [Lebecque and Gearhart, 1990]). This provided evidence of a family of related B cell clones in which C4 exhibited closest homology to the germline and C2, C3 and C8 appeared to have matured and expanded from C4 with the acquisition of further somatic mutations.

The second family isolated from the nasal mucosa of SL5 exhibited a greater diversity in the mutations accumulated by its component clones, although every member expressed the V_H4 gene mapped to VH4-30.1/4-31, the same D gene sequence (the extent of mutation meant the germline gene could not be reliably identified) and JH4b (*Fig.* 4.2.1 B). While the extent of differences between the clones that formed the two main branches of the V_H4 family was such that there was a possibility that the family could, in fact, be divided into two small families (C9 and C11, C13 and C18), careful analysis suggested that they were more likely to be one family of related clones which had diverged significantly as each clone exhibited the same D and J_H genes recombined in the same fashion. The hypothetical intermediate detailed in *Fig.* 4.2.1 B represents the clone that must have accumulated the six V_H region mutations shared between the two branches of the family, but which was not isolated experimentally. C13 exhibited greatest sequence homology with the hypothetical intermediate, sharing the six V_H mutations, but exhibiting an additional five V_H and two J_H mutations. C18 had accumulated a further three V_H mutations. From the other branch of the clonal family, C9 contained twelve additional V_H region mutations and four additional J_H region mutations compared to the hypothetical intermediate. C11 exhibited a single additional mutation to that of C9.

When a PBMC sample from SL5 was examined, evidence of related IgE^+ B cell clones was also observed. Twenty V_H-Cε PBMC sequences were analysed, of which thirteen

were different. All of the clones could be classified into one of three distinct B cell clonal families (*Fig.* 4.2.2 A, B and C). The V_H1 family (VH1-69, an unidentified D gene and JH4b) consisted of two clones; C16 exhibited sixteen mutations from the germline and C13, which had accumulated one further mutation (*Fig.* 4.2.2 A). The second family of B cells expressed VH3-09, D5-24 and JH3b and consisted of eight related clones; C7 exhibited eight V_H mutations from the germline. Seven further clones appeared to be related to C7, each exhibiting one or two additional mutations (*Fig.* 4.2.2 B). A further family (VH3-48, D1-26, JH4b) comprised three clones; C6 differed by twenty-six V_H mutations from the germline, while C2 exhibited one additional mutation and C12 a further mutation compared to C2 (*Fig.* 4.2.2 C).



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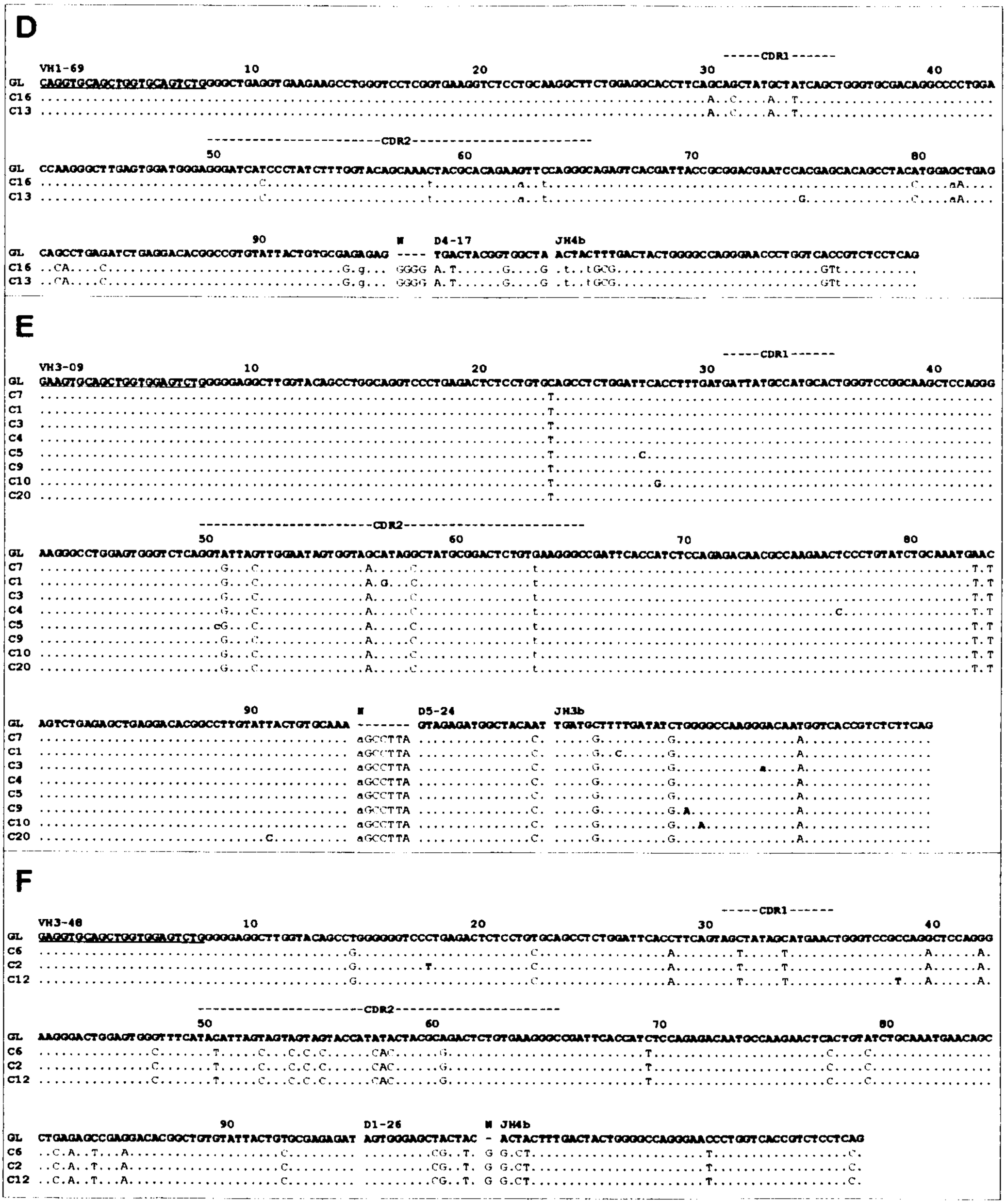


Fig. 4.2.2; Families of IgE⁺ related B cell clones isolated from the PBMC of patient SL5 and the V_H-D-J_H sequences. **A)** V_H1 family expressing genes mapped to the locus VH1-69, an unidentified D gene and JH4b. **B)** V_H3 family expressing VH3-09, D5-24 and JH3b. **C)** V_H3 family expressing VH3-48, D1-26 and JH4b. In A), B) and C) the closest germline homology as determined by VBase is depicted in a black box. Related clones are depicted as ovals. The N region constitutes non-templated nucleotide insertions. Mutations in the V_H region are detailed such that 75(1) represents a mutation at codon 75, position 1. Additional mutations in the D and J_H regions are also detailed. In **D), E) and F)** the sequences of the V_H1, V_H3-09 and V_H3-48 families are detailed respectively. GL represents the closest germline homology as determined by VBase. Primer binding regions are underlined and no mutations occurring in these regions are included. Homology to the germline is represented with a dot, whereas mutations from the germline are depicted either in upper case (if causing an amino acid replacement) or lower case (if having a silent effect upon the amino acid). When the link between clones is dependent on one or two mutations, these are highlighted in red. The CDR regions are detailed.

The sequence data from SL5 appeared to suggest that related IgE^+ B cells existed within the nasal mucosa and also in the peripheral blood. The presence of closely related B cells in the tissue might suggest that local SHM and clonal expansion had taken place (resulting in distinct families of closely related clones). It was however slightly surprising that *all* of the clones could be grouped into a B cell clonal family. More importantly, the apparent extent of related B cell clones in the peripheral blood cast doubt over the entire experiment and prevented such conclusions of local SHM and clonal expansion in the nasal mucosa being justified. If the data was reliable, then an extremely restricted repertoire of IgE^+ B cells must have existed in the blood. Concern, particularly over the apparent clonality of cells isolated from PBMC prompted a more detailed analysis of the data.

SHM typically introduces nucleotide substitutions in which transitions (especially C→T and G→A) are favoured over transversions [Betz *et al.*, 1993b], [Insel and Varade, 1994]. It was evident however, that in both the nasal biopsy and PBMC samples from SL5, the mutations that determined the relatedness of the clones were (with the exception of the multiple mutations differing between the C9 and C13 branch of the VH4-30.1/4-31 family in the nasal mucosa) biased *completely* towards those between C and T or A and G.

Taq DNA polymerase, used habitually in this and previous such PCR amplification of V_H-D-J_H transcripts (*e.g.* [Snow *et al.*, 1997], [Snow *et al.*, 1999], [Van der Stoep *et al.*, 1993]) introduces errors during nucleotide incorporation because of its inability to proofread. The error rate for *Taq* is generally accepted to vary between 1.1×10^{-4} and 8.9×10^{-5} mutation frequency per base pair per duplication (mut bp^{-1} dup^{-1}) depending on the experimental systems in which it is used [Tindall *et al.*, 1988], [Barnes, 1992], [Cariello *et al.*, 1991]. *Taq* also has a tendency to cause errors that are nucleotide transitions rather than transversions, especially T→C and A→G [Rotherfluh *et al.*, 1993], [Zylstra *et al.*, 1998]. It therefore seemed likely that the mutations observed in samples from SL5 and on the basis of which the families of related B cell clones had been constructed, may have resulted in part or completely as a result of errors incorporated by *Taq*.

Assuming that all of the mutations observed in the B cell clones from SL5 that were responsible for the formation of families of related clones (with the exception of those differing between C9 and C13 in the nasal biopsy) resulted from errors introduced experimentally by *Taq*, the error rate in this experiment was calculated to be 1 in every 630 bp of DNA amplified. This equated to an error rate of 2.7×10^{-5} mut bp^{-1} dup^{-1} in this experimental system, consistent with that detailed previously (see appendix A for all calculations) without taking account of the small contribution to error made by the reverse transcriptase.

If all these mutations were incorporated by *Taq*, the structure of the families would be significantly altered, resulting in the possibility that all of the families observed in the PBMC and all except for the distantly related C9 and C13 in the nasal biopsy sample would be eliminated. This alters the data set significantly, limiting the families in the nasal biopsy to just the one VH4-30.1/4-31 family, with a hypothetical intermediate linking two distantly related B cells. It was evident therefore, that *Taq* DNA polymerase introduced experimental errors at a rate that prevented meaningful interpretation of the data. Whilst the conditions employed in the PCR are known to be able to improve the fidelity of a PCR [Cline *et al.*, 1996], this was not deemed to be sufficient and the use of another DNA polymerase, *Pfu*, was investigated.

Pfu DNA polymerase has a lower error rate (approximately 1.3×10^{-6} mut bp^{-1} dup^{-1}) than *Taq* as a consequence of a proofreading ability conferred by the 3'→ 5' exonuclease activity that it exhibits [Cline *et al.*, 1996]. In order to analyse the exact effect of *Taq* compared to *Pfu* in a more accurate manner, analysis of the number of mutations incorporated into the Cε1 region amplified 3' of each V_H region, a region known to be unaffected by SHM [Lebecque and Gearhart, 1990] was analysed.

On the basis of mutations introduced experimentally into the constant Cε1 region, the error rate for *Taq* DNA polymerase in the amplification of the nasal biopsy and PBMC samples from SL5 was calculated to be approximately 3.4×10^{-5} mut bp^{-1} dup^{-1} (1 mutation for every 487 bp incorporated), confirming that it was likely that all of the mutations observed in the V_H regions amplified from SL5 were introduced experimentally. In contrast, when Cε1 sequences amplified by *Pfu* were analysed,

under conditions optimal for fidelity [Cline *et al.*, 1996], an error rate of 3.5×10^{-6} mut bp^{-1} dup^{-1} (1 mutation every 4720 bp) was determined.

While *Pfu* exhibited a much greater accuracy than *Taq* in the experimental system, an error rate of 3.5×10^{-6} mut bp^{-1} dup^{-1} is not as low as that observed by other researchers (1.3×10^{-6} mut bp^{-1} dup^{-1} [Cline *et al.*, 1996] and $0.9 - 1.0 \times 10^{-6}$ mut bp^{-1} dup^{-1} [Slater *et al.*, 1998]). Differences in the observed error rate compared to those stated in the literature may have resulted from the inevitable inaccuracies incurred in attempting to quantitate a nested PCR.

It was decided on the basis of the error rates exhibited by the DNA polymerases that *Pfu* would be used in all future experiments in order to enable the confident interpretation of data, although it was noted that 1 mutation approximately every 4720 bp (17 V_H-D-J sequences) was likely to result from experimental error.

4.3 Analysis of V_H-Cε sequences amplified by *Pfu* from the nasal mucosa and PBMC of a cohort of seven allergic rhinitis patients.

In order to continue investigating whether local SHM and clonal expansion occurred locally in the nose, nasal biopsy and for comparison, PBMC samples, were studied from a cohort of seven allergic rhinitis patients, three male and four female, aged between eighteen and thirty-eight. While all seven patients were allergic to *Phleum pratense* (grass pollen), six were allergic to multiple aeroallergens and only one (HD17) was grass pollen mono-allergic (as determined by skin-prick test and RAST). All the patients selected for this study exhibited a total serum IgE > 200 IU / ml (in order to increase the probability of successful PCR amplification of V_H-Cε). The samples were taken randomly throughout the year, such that some samples were taken within and some outside the grass pollen season (Table 4.3.1). Nasal biopsy and PBMC samples were not taken from normal subjects for control purposes, because normal IgE even in the serum, is prohibitively low.

Patient	Age	Sex	Status at biopsy [a)]	Total serum IgE (IU / ml) (Norm = 3 - 150)	Specific serum IgE (IU / ml) [b)] (Norm < 0.35)	Allergies [c)]
CD6	19	M	I / P	990	HDM = 1.28 G = 72.9 T = 101	HDM, G, M, T, B, C, D
JB7	37	M	O / P	236	G = 9.25 C = 11.1	HDM, G, C, D, CL
CM10	18	F	O / P	382	HDM = 1.09 G = 101 C = 3.36	HDM, G, M, T, B, C, D, H, CL
HD14	21	F	I / P	787	HDM = 2.68 G = 101	HDM, G
SO16	37	M	I / P	514	G = 101	G, T, B, D
HD17	22	F	I	2745	G = 101 C = 0.39	G
AP19	38	F	I / P	414	HDM = 1.23 G = 60.2 C = 32.1	HDM, G, C, D, H, CL

Table 4.3.1: Clinical data from allergic rhinitis patients CD6, JB7, CM10, HD14, SO16, HD17 and AP19. ***a)*** In-season (I), out-of-season (O), perennial (P). **b)** Determined by RAST for the allergens; house dust mite (HDM), mixed grass (G), tree(T), cat (C). **c)** Determined by a skin-prick test of ≥2 mm diameter as; *Dermatophagoides* (house dust mite) (HDM), *Phleum pratense* (grass pollen) (G), mugwort (M), three trees (T), silver birch (B), cat (C), dog (D), horse (H), *Aspergillus fumigatus* (AF), *Cladosporium herbarum* (CL), *Alternaria alternata* (A).

V_H-Cε sequences were amplified from each nasal biopsy and PBMC sample with *Pfu* DNA polymerase in order to minimise the introduction of experimental errors (as discussed in section 4.2 and according to the methods detailed in section 3.2.18). The PCR products from each sample were subjected to agarose gel electrophoresis (*Fig.* 4.3.1), extracted from the agarose gel, cloned and fifteen sequences analysed from almost every sample (see Tables 4.3.2 and 4.3.3 for details), according to the methods described in sections 3.2.26 - 36. The analysis of fifteen sequences was chosen as an appropriate sample size on the basis that when a further ten sequences, generated from a

sample PCR were analysed, only one further unique sequence was identified (data not shown).

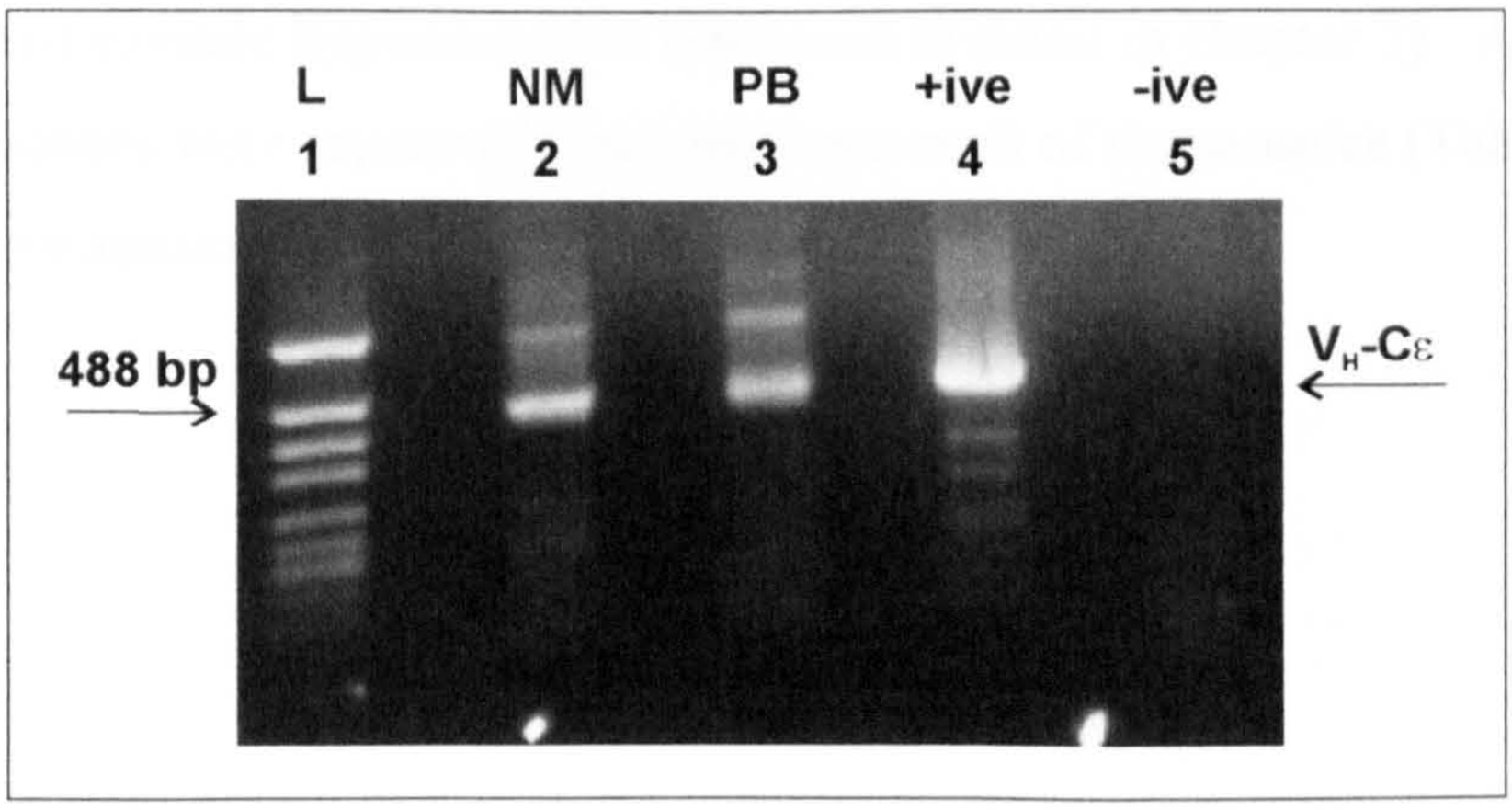


Fig. 4.3.1; Agarose gel electrophoresis of V_H-Cε PCR products amplified from the nasal mucosa and PBMC of allergic ***rhinitis patient CD6.*** A typical example of V_H-Cε PCR products (indicated at approximately 450 bp) subjected to agarose gel electrophoresis on a 1% agarose gel and visualised under UV. **Lane 1**; *Hpa II* DNA ladder (L), (488bp indicated). **Lane 2**; V_H-Cε PCR products amplified from the nasal mucosa of patient CD6 (NM). **Lane 3**; V_H-Cε PCR products amplified from PBMC of patient CD6 (PB). **Lane 4**; V_H-Cε PCR positive control (AF10 cells). **Lane 5**; V_H-Cε PCR negative control (no DNA).

V_H genes from each of the V_H1 to V_H7 classes, with the exception of V_H2; one of the most infrequently expressed V_H classes [Brezinschek *et al.*, 2000], were amplified from different samples. Independent experiments demonstrated that V_H2 and Cε primers were successfully able to amplify V_H2 from a PBMC sample, although it is possible that the primers worked with decreased efficiency compared to those for the other V_H gene classes.

All sequences demonstrated rearranged V_H-D-J_H genes expressing diversity across the junctions, including in many cases the insertion of non-templated nucleotides (N) at the V_H-D or D-J_H junction. With the exception of two distinct clones (C1 and C13 isolated from the PBMC of CM10), all of the V_H-Cε sequences contained mutations in the V_H region. The average degree of V_H gene mutation in sequences isolated from the nasal biopsy samples was 6.6% (range 1.8 – 22.7%) compared to 5.5% in the PBMC (range 0 – 11.7%) (see Tables 4.3.2 and 4.3.3). The slightly increased average (and range) of somatic hypermutation in sequences from the nasal biopsies possibly reflecting increased affinity maturation by SHM in response to (perhaps local) allergen exposure.

The general pattern of mutations observed in the sequences isolated from both the nasal biopsy and PBMC samples was consistent with that previously observed to be characteristic of somatic hypermutation (analysed in detail in chapter 7). As previously, identical sequences were repeatedly isolated from each of the samples (Tables 4.3.2 and 4.3.3), but were assumed to have resulted from one clone.

Patient	Unique sequences isolated from nasal biopsy samples	Predicted germline V_H gene usage	% Mutation from germline	Number of identical sequences isolated
CD6	C2	3-23	6.2	2
	C12	3-30/3-30.5	5.2	1
	C1	3-30.3	4.0	6
	C5	3-33	4.0	4
	C13	6-01	5.7	2
JB7	C14	1-46	22.7	1
	C9	3-23	5.5	1
	C17	3-74	4.8	8
	C22	4-04	7.3	5
CM10	C7*	3-30/3-30.5	2.9	1
	C13	3-30/3-30.5	4.4	1
	C1	3-66	10.9	1
	C2	3-66	10.7	12
HD14	C16	1-02	5.9	1
	C9	3-15	9.9	1
	C4	3-30	11.0	1
	C2	3-30	7.0	8
	C11	4-59	6.4	1
	C19	5-a	4.5	1
	C7	5-51	7.4	1
	C12	5-51	4.8	1
SO16	C12	3-20	3.3	2
	C18	3-23	1.8	1
	C2**	3-30	4.1	1
	C3**	3-30	5.2	2
	C10	3-33	5.6	2
	C1	3-65	ND	1
	C13	4-39	3.7	1
	C5*	5-51	2.9	1
	C8	5-51	8.1	1
	C11	5-51	7.3	1
	C14	5-51	7.3	2
HD17	C13	1-69	4.8	1
	C1	3-30	9.6	1
	C16	4-ND	12.1	1
	C9	4-30.1/4-31	8.4	1
	C37	4-30.1/4-31	8.8	1
	C15*	4-30.2	7.7	5
	C31	4-30.2	8.5	3
	C32	4-59	5.6	1
	C7	7-04.1	3.7	1
AP19	C16	1-18	2.2	1
	C6	4-04	5.2	1
	C3	4-30.1/4-31	2.5	2
	C2	5-51	7.1	8
	C4	5-51	6.4	1
	C8	5-51	ND	1
	C14	5-51	7.5	1

Table 4.3.2: V_H gene usage and % V_H mutation of V_H-Cε sequences isolated from nasal biopsies from allergic rhinitis patients CD6, JB7, CM10, HD14, SO16, HD17 and AP19. V_H gene usage is detailed such that a gene mapped to the locus 5-51 is a V_H5 gene. V_H genes that could not be determined are denoted ND. Sequences highlighted in red represent related clones isolated from that patient (or in yellow where more than one family of related clones was isolated from the same sample). Where an identical sequence was isolated from the patient's corresponding PBMC sample it is marked with an asterisk. Where a sequence representing a distantly related clone was isolated from the patient's corresponding PBMC sample it is marked by two asterisks (see Table 4.3.3).

Table 4.3.3: V_H gene usage and % V_H mutation of V_H-Cε sequences isolated from the PBMC of allergic rhinitis patients CD6, JB7, CM10, HD14, SO16, HD17 and AP19. V_H gene usage is detailed such that a gene mapped to the locus 5-51 is a V_H5 gene. V_H genes that could not be determined are denoted ND. Sequences highlighted in red represent related clones isolated from that patient (or in yellow where more than one family of related clones was isolated from the same sample). Where an identical sequence was isolated from the patient's corresponding nasal biopsy it is marked with an asterisk. Where a sequence representing a distantly related clone was isolated from the patient's corresponding nasal biopsy it is marked by two asterisks (see Table 4.3.2).

Patient	Unique sequences isolated from PBMC samples	Predicted germline V_H gene usage	% Mutation from germline	Number of identical sequences isolated
CD6	C2b	1-18	5.5	1
	C28	3-30.3	4.0	2
	C33	3-33	2.6	1
	C1	4-30.2	3.7	10
	C4b	6-01	5.7	1
JB7	C2	1-18	4.4	2
	C17	3-30/3-30.5	7.0	2
	C3	3-30/3-30.5	4.0	3
	C18	3-73	8.3	2
	C4	3-73	8.7	2
	C16	5-a	3.0	2
CM10	C13	3-23	0	1
	C3*	3-30/3-30.5	2.9	13
	C1	3-30.3	0	1
HD14	C2	1-18	7.0	1
	C17	1-18	6.6	1
	C4	3-30	4.8	3
	C16	3-30	3.7	2
	C3	4-04	6.6	2
	C1	4-04	5.5	1
	C8	4-30.1/4-31	6.2	2
	C13	4-39	1.8	2
	C7	5-a	10.0	1
SO16	C5	1-08	7.0	1
	C2	3-23	4.7	2
	C10	3-30	3.3	1
	C3	3-30	1.9	2
	C4	3-33	5.2	1
	C7	3-33	6.7	1
	C1	4-39	ND	1
	C8	5-51	8.1	1
	C11	3-09	6.7	1
	C9**	3-30	5.6	1
	C14	3-33	4.8	1
	C15*	5-51	2.6	1
HD17	C9	1-09	6.7	1
	C28	3-30	6.7	1
	C12	3-30	4.1	1
	C1	3-30	6.7	1
	C18	3-30	5.9	1
	C23	3-66	4.4	1
	C15	4-04	7.4	1
	C7	4-30.1/4-31	2.6	2
	C11*	4-30.2	7.7	1
	C14	4-30.2	8.1	1
	C6	7-04.1	11.4	1
	C25	7-04.1	11.7	1
	C24	7-04.1	7.3	2
AP19	C20	3-30	2.9	1
	C19	3-13	8.6	1
	C7	3-33	4.0	1
	C8	4-30.1/4-31	3.6	1
	C16	4-30.1/4-31	2.5	1
	C15	4-30.1/4-31	2.9	1
	C1	4-61	10.3	3
	C10	5-51	7.0	2
	C2	6-01	9.6	4

When sequences isolated from each sample were analysed, the presence of families of related B cell clones (with identical signature regions, but diverse V_H mutations) was apparent in four of the six nasal biopsy samples (CM10, SO16, HD17 and AP19) and three of the six PBMC samples (JB7, HD17 and AP19), (*Fig.* 4.3.2 and 4.3.3). No families of related B cells were isolated from either the nasal biopsy or PBMC samples of CD6 and HD14.

In addition, sequence analysis suggested that in each of three patients (CM10, HD17 and SO16) a B cell clone(s) with the same signature region to that isolated from the nasal mucosa was present in the patient's PBMC (see Tables 4.3.2 and 4.3.3). In both CM10 and HD17 the clones in the nasal mucosa and PBMC were shown to be identical. However in patient SO16, while one identical clone to that in the nasal mucosa was isolated from the PBMC sample, an additional clone was isolated from the PBMC that was distantly related to the V_H3 family of B cell clones isolated from the nasal mucosa (*Fig.* 4.3.4). This PBMC clone (PC9) exhibited multiple distinct mutations throughout the V_H region in addition to mutations shared with the related family of B cell clones in the nasal mucosa.

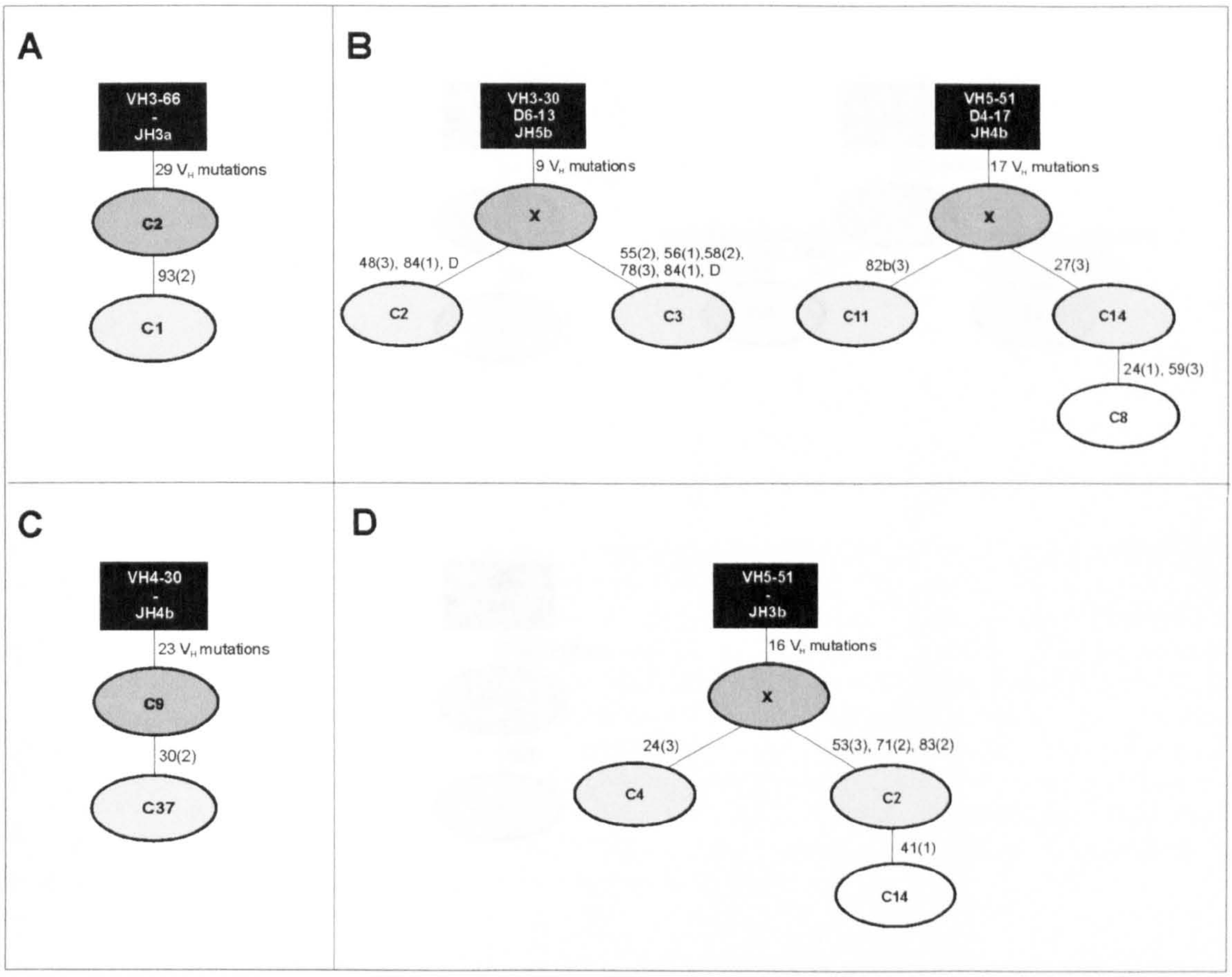


Fig. 4.3.2; Families of related IgE⁺ B cell clones isolated from the nasal mucosa of allergic rhinitis patients A) CM10, B) SO16, C) HD17 and D) AP19. **A)** CM10; V_H3 family expressing genes mapped to the locus VH3-66, an unidentified D gene and JH3a. **B)** SO16, two families; a V_H3 family expressing VH3-30, D6-13 and JH5b and a V_H5 family expressing VH5-51, an unidentified D gene and JH4b. **C)** HD17; V_H4 family expressing VH4-30, an unidentified D gene and JH4b. **D)** AP19; V_H5 family expressing VH5-51, an unidentified D gene and JH3b. In A) to D) the closest germline homology as determined by VBase is depicted in a black box. Related clones are depicted as ovals. Hypothetical clones, not isolated experimentally but which must be intermediates are denoted X. Mutations are detailed such that 93(2) represents a mutation at codon 93, position 2. Additional mutations in the D region are also detailed.

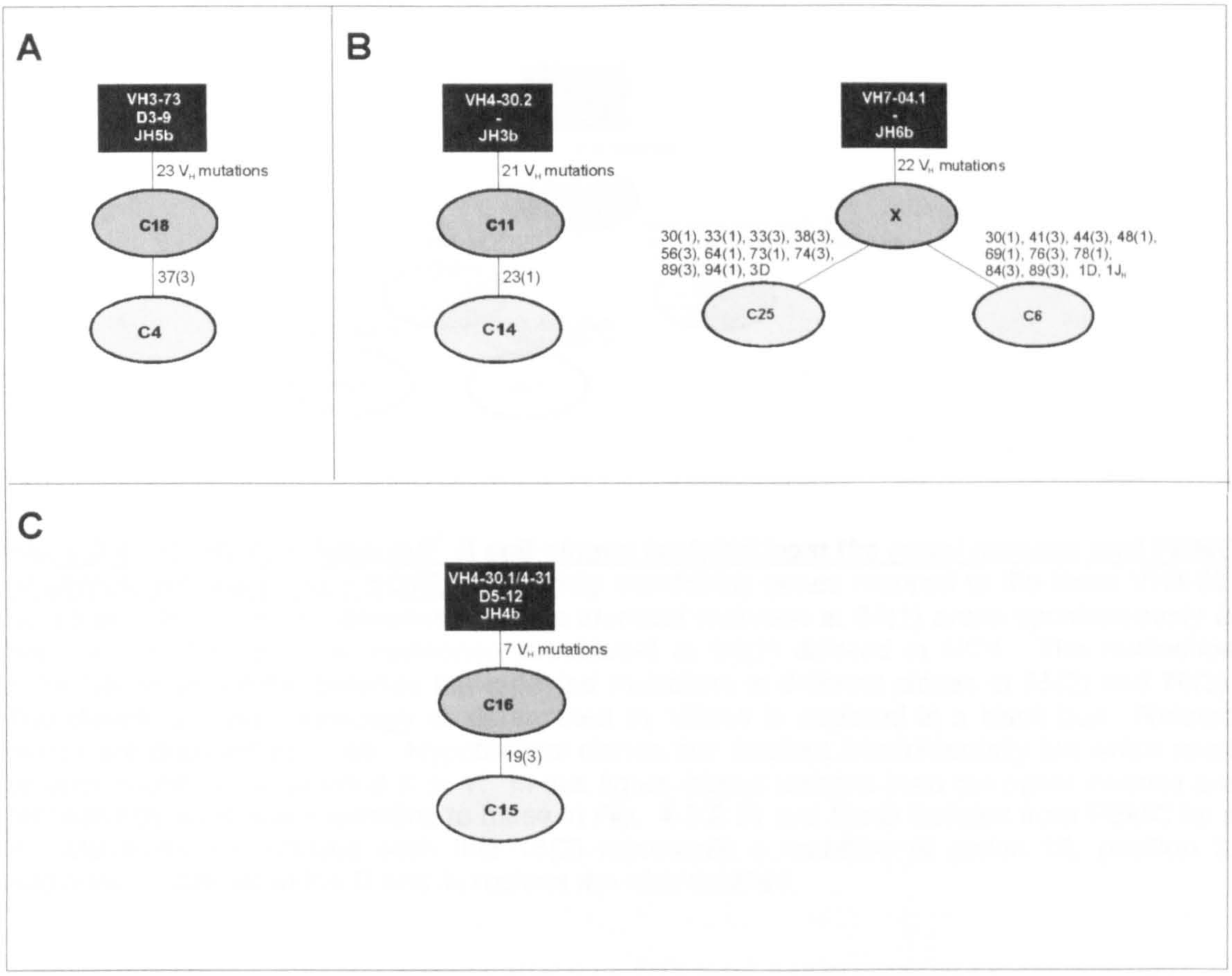


Fig. 4.3.3; Families of related IgE$^+$ B cell clones isolated from the PBMC of allergic rhinitis patients A) JB7, B) HD17 and C) AP19. **A)** JB7; V_H3 family expressing genes mapped to the locus VH3-73, D3-9 and JH5b. **B)** HD17; two families; a V_H4 family expressing VH4-30.2, an unidentified D gene and JH3b and a V_H7 family expressing VH7-04.1, an unidentified D gene and JH6b (and in which the nucleotide substitutions at 30(1) and 89(3) differ between C6 and C25). **C)** AP19; V_H4 family expressing VH4-30.1/4-31, D5-12 and JH4b. In A), B) and C) the closest germline homology as determined by VBase is depicted in a black box. Related clones are depicted as ovals. Hypothetical clones, not isolated experimentally but which must be intermediates are denoted X. Mutations are detailed such that 37(3) represents a mutation at codon 37, position 3. Additional mutations in the D and J_H regions are also detailed.

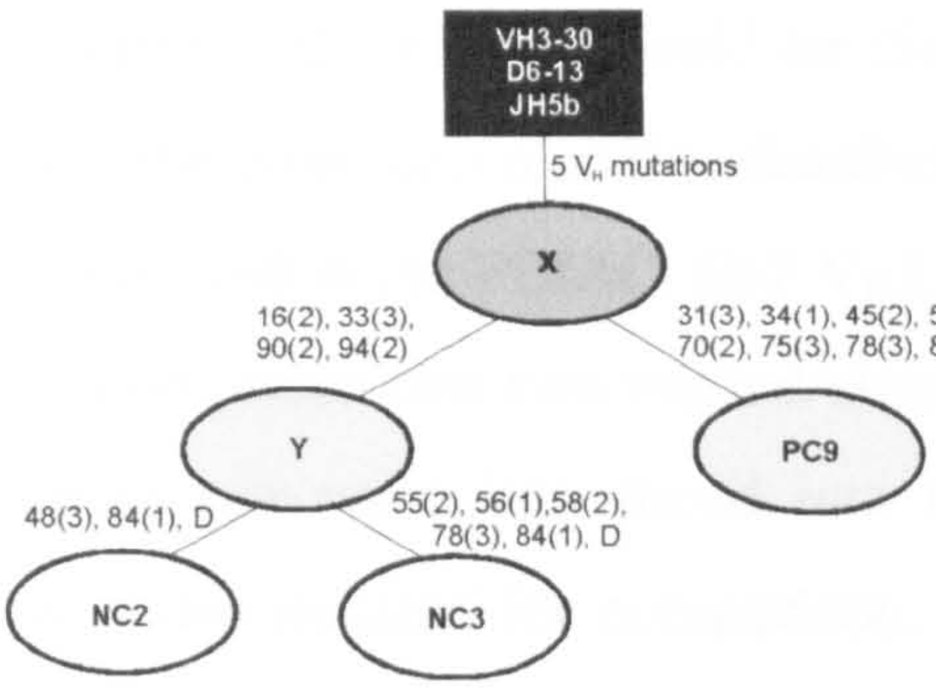


Fig. 4.3.4; Family of related IgE⁺ B cell clones isolated from the nasal mucosa and PBMC of allergic rhinitis patient SO16. V_H3 family expressing genes mapped to the locus VH3-30, D6-13 and JH5b. It was assumed that the identical mutation at 84(1) arose spontaneously in both NC2 and PC9. The nucleotide substituted at 84(1) differed in NC3. The nucleotide substitutions also differ between the repeated mutations in different clones at 55(2) and 78(3). The closest germline homology as determined by VBase is depicted in a black box. Related clones are depicted as ovals. Hypothetical clones, not isolated experimentally but which must be intermediates are denoted X or Y. In this figure clones isolated from the nasal mucosa are preceded by an N (corresponding to those in *Fig.* 4.3.2 B) and those isolated from PBMC by a P. Mutations are detailed such that 16(2) represents a mutation at codon 16, position 2. Additional mutations in the D and J_H regions are also detailed.

As discussed in section 4.2, even with the use of *Pfu* DNA Polymerase, the experimental error rate determined for this system was shown to be approximately 1 in 4720 bp. As the number of bases analysed in each V_H region is approximately 270 bp (excluding the primer region and depending on the gene and any polymorphisms), this equates to approximately 1 mutation being artificially generated in the V_H region of approximately every 17.5 sequences generated. From this cohort of seven patients a total of 207 sequences were analysed from the nasal biopsy and PBMC. Assuming the error rate did not vary between the samples, this implies that eleven or twelve mutations would be predicted to result from experimental error amongst the 207 sequences studied, affecting eleven or twelve sequences (assuming an even distribution of such mutations). However, such errors would only be identifiable, if an identical sequence that had not been subject to experimental error, was also isolated from the sample for comparison, this in turn being heavily dependent upon the cycle number of the PCR in which the error was introduced.

In order to ensure that data resulting from experimental errors did not influence the validity of the results presented in this thesis, it was decided that any sequences that

implied the existence of families of related B cell clones on the basis of only one nucleotide difference between the clones would be discarded for the purposes of data analysis. This resulted in the elimination of five families of B cells (in the nasal mucosa; CM10 V_H3 and HD17 V_H4 and in the PBMC; JB7 V_H3, HD17 V_H4 and AP19 V_H4). As each eliminated family comprised just two related clones, this implied five instances in which experimental mutations were introduced into the V_H region and in which an unmutated sequence was also isolated for comparison. The remaining reliable families of B cell clones, in which the basis of the families was not solely dependent upon a single nucleotide difference are summarised below.

Sequences indicating the presence of three reliable families of related IgE^+ B cell clones were isolated from the nasal mucosa of two of the seven allergic rhinitis patients studied (two families from SO16 (*Fig.* 4.3.2 B) and one family from AP19 (*Fig.* 4.3.2D)) and are summarised in *Fig.* 4.3.5. Two related B cells in one family from nasal biopsy SO16 expressed the genes VH3-30, D6-13 and JH5b and shared nine V_H mutations with a hypothetical intermediate whilst differing from each other by seven V_H and two D region mutations. The second family isolated from nasal biopsy SO16 consisted of three related B cell clones each expressing VH5-51, D4-17 and JH4b. These clones were linked by a hypothetical intermediate with which all the clones shared seventeen mutations. Both C11 and C14 had one additional mutation to the hypothetical intermediate and the third clone, C8 differed from C14 by a further two mutations. The family of related B cells isolated from AP19 comprised three related clones, all of which shared sixteen V_H mutations with a hypothetical intermediate. C4 differed from the hypothetical intermediate by one further mutation, while C2 differed by three further mutations. C14 exhibited an additional mutation compared to C2.

In each of these reliable families isolated from nasal biopsies SO16 and AP19, while the relationship of a B cell with its sister clones may be altered if a single individual mutation were demonstrated to be an experimental artefact, the clone would in each case take another position within the family, for example, in the VH5-51 family from isolated from the nasal mucosa of SO16, if the single mutation at 82b(3), linking C11 to the rest of the family were discredited, C11 would move to take the place of the hypothetical intermediate. Such families were not therefore eliminated from the data set.

The presence of only one reliable family of related B cell clones (VH7-04.1 in *Fig.* 4.3.3 B) was isolated from the PBMC (HD17). This family consisted of two related B cell clones; C6 and C25 shared twenty-two V_H mutations with a hypothetical intermediate, but differed by nineteen V_H mutations. In addition, whilst a distinct signature region was shared by the two clones, four D region mutations (two of which included mutation of the same nucleotide) and one J_H mutation differed between the clones. In view of the slight variation at the D-J_H junction, the likelihood of C6 and C25 being related was strengthened by the number of V_H region mutations shared between these clones. No other reliable related B cell clones were isolated from the PBMC of any of the other patients in the cohort.

There was one reliable example of a clone isolated from the PBMC and yet related to a family of related B cells from the nasal biopsy (SO16, *Fig.* 4.3.4). The PBMC clone (PC9) shared junctional homology and five V_H mutations with the family of B cells isolated from the nasal mucosa and that expressed the genes VH3-30, D6-13 and JH5b. PC9 differed by ten V_H mutations, one D and two J_H mutations from the hypothetical intermediate that linked it with other members of the clonal family. The J_H region mutations were distant to the D-J junction, located in the body of the J_H segment.

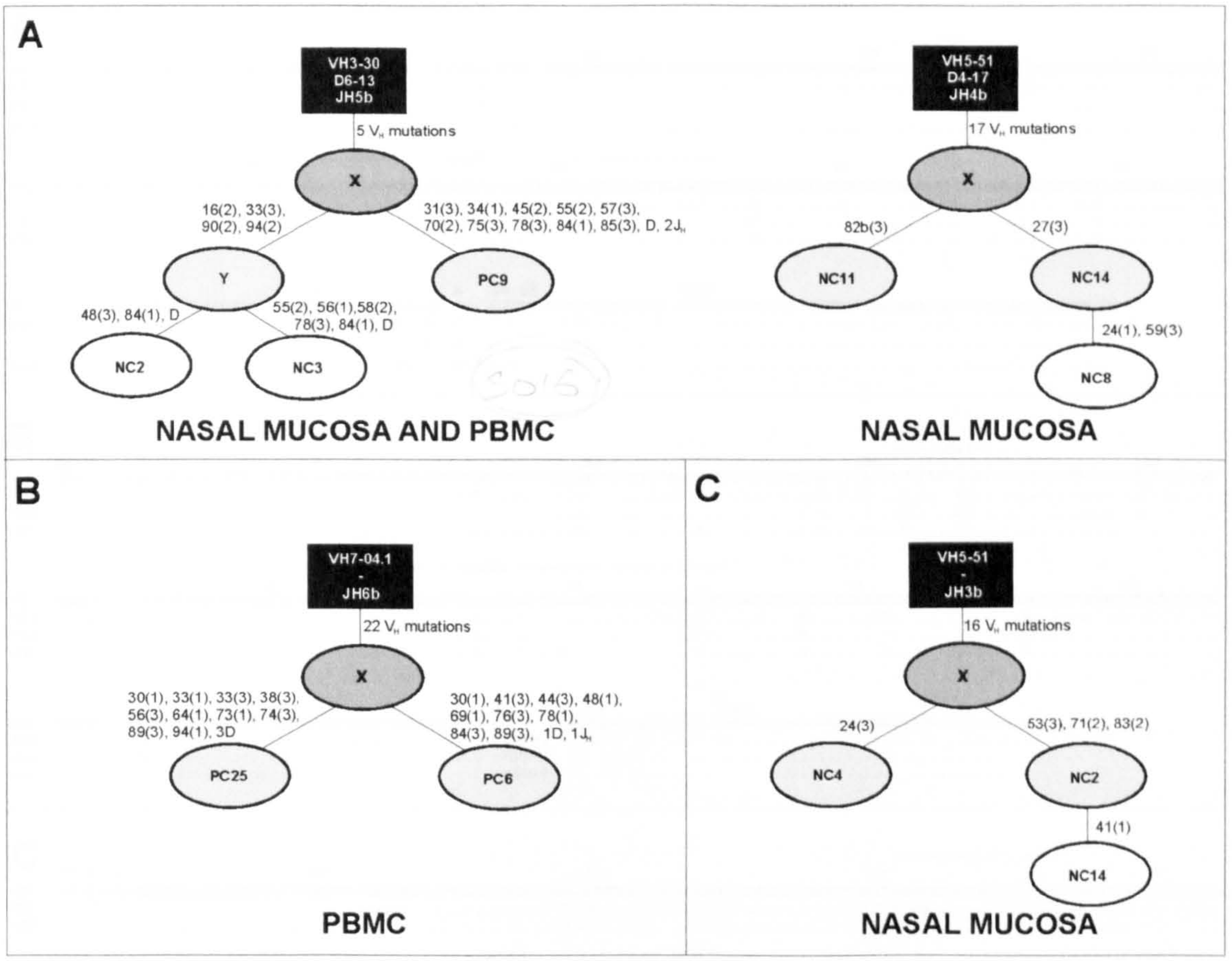


Fig. 4.3.5; Families of related IgE$^+$ B cell clones generated from reliable data from either the nasal mucosa or PBMC of allergic rhinitis patients A) SO16, B) HD17 and C) AP19. **A)** SO16, two families; a V_H3 family of two clones from the nasal mucosa and one related clone from the PBMC expressing VH3-30, D6-13 and JH5b. The V_H5 family of related clones in SO16 were all isolated from the nasal mucosa and expressed VH5-51, an unidentified D gene and JH4b. **B)** HD17; V_H7 family of clones isolated from the PBMC expressing VH7-04.1, an unidentified D gene and JH6b. **C)** AP19; V_H5 family of clones all isolated form the nasal mucosa and expressing VH5-51, an unidentified D gene and JH3b. The closest germline homology as determined by VBase is depicted in a black box. Related clones are depicted as ovals. Clones isolated from the nasal mucosa are denoted NC and those from the PBMC, PC. The hypothetical clones, not isolated experimentally but which must be intermediates are denoted X or Y. Mutations are detailed such that 16(2) represents a mutation at codon 16, position 2. Additional mutations in the D and J_H regions are also detailed.

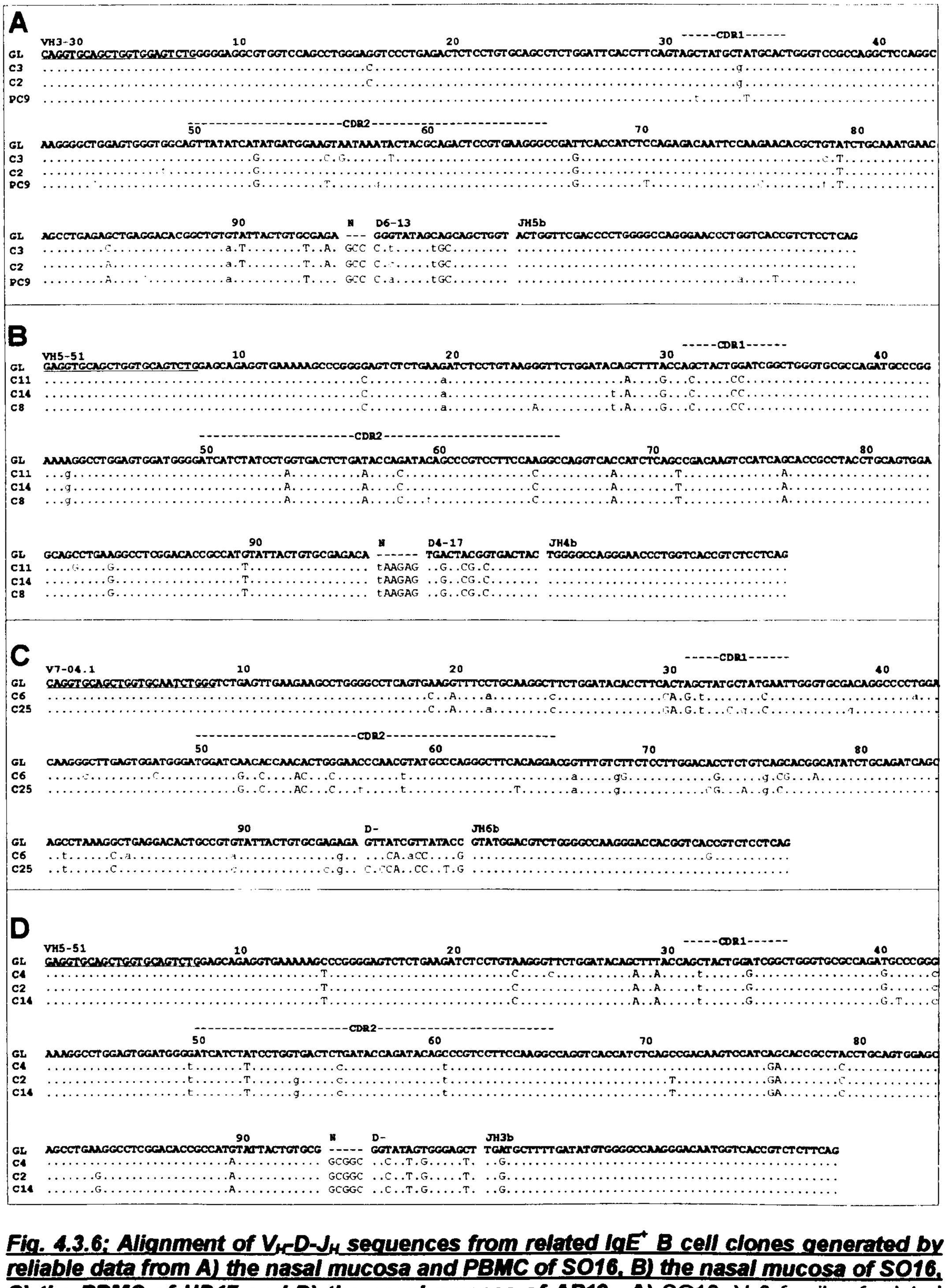


Fig. 4.3.6; Alignment of V_H-D-J_H sequences from related IgE$^+$ B cell clones generated by reliable data from A) the nasal mucosa and PBMC of SO16, B) the nasal mucosa of SO16, C) the PBMC of HD17 and D) the nasal mucosa of AP19. **A)** SO16; V_H3 family of related clones (including PC9 from PBMC) expressing the genes VH3-30, D6-13 and JH5b. **B)** SO16; V_H5 family expressing VH5-51, D4-17 and JH4b. **C)** HD17; V_H7 family expressing VH7-04.1, an unidentified D gene and JH6b. **D)** AP19; V_H5 family expressing VH5-51, an unidentified D gene and JH3b. GL represents the closest germline gene homology as determined by VBase. Primer binding regions are underlined and no mutations in these regions included. Homology to GL is represented by a dot, mutations from GL are in upper case (if a replacement amino acid mutation) or lower case (if a silent amino acid mutation). Mutations that determine families are depicted in red. CDR regions are detailed.

It is of interest that the three reliable families of related IgE^+ B cell clones isolated from the nasal mucosa samples SO16 and AP19 were both from biopsies taken within the grass pollen season, although related IgE^+ B cell clones were not isolated from the other nasal biopsy samples also taken in-season (CD6, HD14 and HD17), preventing any complete correlation with the presence of related clones in the nasal mucosa and the time at which the nasal biopsies were taken. Unfortunately, no out-of-season biopsies were taken from grass pollen mono-allergic patients for comparison. There was also no apparent correlation between the number of different clones isolated from a sample and the likelihood of detecting a family of related clones from that sample, as while a comparatively wide range of different clones were isolated from SO16 and AP19, similar numbers of different clones were isolated from biopsy samples in which no related clones were detected *e.g.* HD14. SO16 and AP19 were allergic to multiple allergens, sharing only an allergy to grass and dog. Exposure of the patients to non-grass allergens could not be assessed. Both patients were within the middle of the range of total serum IgE values exhibited by the cohort of patients. It was notable that of the three families of clones isolated from the nasal mucosa, two of the families comprised clones expressing V_H5-51 (discussed in greater detail in chapter 7).

In each of the three B cell clonal families isolated from the nasal mucosa the relationship between the clones was very close, with one hypothetical intermediate needed to link each set of related clones. The presence of these clones indicates that it is likely that local SHM and clonal expansion occurred in the nasal mucosa, in order that clones with such a close degree of relatedness were generated in such a small area (approx. 2.5 mm^3). The alternative to this, the individual migration of each of the related clones from the lymphoid tissue (which could not have been inadvertently sampled, the nearest being Waldeyer's Ring in the pharynx, approximately 8 – 10 cm distal to the inferior turbinate) to the same small area of the inferior turbinate sampled by biopsy, is unlikely.

In contrast to the three families of closely related IgE^+ B cells isolated from the nasal mucosa, only one family of related IgE^+ B cell clones (patient HD17) was isolated from the corresponding seven PBMC samples examined. This PBMC family also incorporated a hypothetical intermediate, but comprised two clones that were more distantly related than any of the clones in the nasal mucosa. While no families were isolated from the nasal biopsy of patient HD17, HD17 exhibited by far the highest total

serum IgE value in the cohort. In addition, HD17 was determined to be monoallergic to grass by skin-prick testing (although RAST results suggested that while the hugely predominant quantity of specific IgE was directed against grass, a minor quantity of anti-cat IgE was also present). It is possible that there was an increased chance of detecting related PBMC clones from patient HD17 because of the nature of the IgE repertoire they expressed, directed in significant manner towards restricted antigens (although the number of clones exhibiting different V_H regions did not appear restricted compared to other patients).

The detection of identical clones from the nasal biopsy and PBMC sample of CM10, SO16 and HD17 implies that, unless cross-contamination between nasal biopsy and PBMC occurred in each set of samples, identical sister B cell clones may move between the nasal mucosa and PBMC. It is impossible to determine whether the clones isolated in the PBMC resulted from the 'overspill' of locally generated B cell clones in the nasal mucosa, or whether they were examples of B cells subjected to SHM and clonal expansion in the lymphoid tissue, which then migrated independently into the blood and *via* the blood, into the nasal mucosa. There would seem to be no evidence to exclude the possibility that some individual IgE^+ B cells in the nasal mucosa result from the conventional routes of maturation and migration, while other families of clonally related IgE^+ B cells are generated locally in the mucosa. Alternatively, while it would seem unlikely, it is possible that a clone detected in the nasal biopsy was identical to a clone from the PBMC because it resulted from a PBMC present within the nasal biopsy itself.

In SO16, the only sample in which clones distantly related in both the nasal mucosa and PBMC were detected, the PBMC clone (PC9) was related to the V_H3 family of B cell clones from the nasal mucosa. The branching of PC9 from the family occurred early in the evolution of the clonal family, as it was much more distantly related than its sister clones isolated from the nasal mucosa. It is again possible that clones migrated independently into both the nasal mucosa and peripheral blood from the lymphoid tissue, and that the related B cell clones isolated from the nasal mucosa were then generated locally. It is also possible that PC9 was generated in the nasal mucosa and migrated into the blood circulation.

It was impossible to determine what type of B cell the sequences generated by PCR originated from, *e.g.* memory cell or plasma cell. It might be assumed that as plasma

cells would produce by far the greatest number of V_H-Cε mRNA transcripts, amplification of sequences may have been biased towards these cells when they were present.

4.4 Analysis of local IgE production in the nasal mucosa of a non-atopic subject with elevated systemic IgE.

In order to increase the efficacy of the PCR reaction, all of the patients selected for this study exhibited total serum IgE values of >200 IU / ml, the highest being 2745 IU / ml exhibited by HD17 (normal range 3 – 150 IU / ml). It was therefore important to determine if the presence of related IgE^+ B cells in the nasal mucosa occurred as a consequence of high total serum IgE rather than as a consequence of allergic rhinitis (*e.g.* by the random migration of the B cells such that they would be present in any tissue). While this explanation seemed unlikely as analysis of the repertoire of B cells in the PBMC of allergic rhinitics did not suggest a close similarity to the B cell repertoire in the nasal mucosa, it was necessary to exclude the possibility, ~~particularly~~ as the lymphoid pool had not been sampled.

Several different experiments would have enabled investigation of the hypothesis that families of related IgE^+ B cells in the nasal mucosa resulted from random migration from a systemic pool of IgE^+ B cells, all of these based on analysis of V_H-Cε sequences and the presence or absence of families of related IgE^+ B cells in the nasal mucosa of subjects exhibiting high total serum IgE, but no respiratory symptoms and in particular no allergic rhinitis. Unfortunately access to a food or bee venom allergic patient with a high total serum IgE but who did not suffer from any respiratory symptoms was not possible. Nor was it possible to gain access to a patient suffering from a gastrointestinal parasitic infection.

A suitable subject, GJ29, was however recruited and a nasal biopsy and PBMC sample obtained. GJ29 reported no history of any allergic reactions or respiratory conditions. In addition, whilst GJ29's childhood was spent in Asia, there was no evidence to suggest recent parasitic infection, although he exhibited a total serum IgE of 1834 IU / ml as determined by RAST (a value in excess of that exhibited by all but one of the allergic rhinitis patients studied). In addition, the skin-prick test showed a negative

reaction to all the tested aeroallergens, although a slightly raised RAST (0.71 IU / ml, compared to the norm of <0.35) to *Aspergillus fumigatus* was noted (Table 4.4.1).

Patient	Age	Sex	Status at biopsy	Total serum IgE (IU / ml) (Norm = 3 - 150)	Specific serum IgE (IU / ml) [a] (Norm < 0.35)	Allergies [b]
GJ29	55	M	Non-atopic	1834	AF = 0.71	None

Table 4.4.1: Clinical data from non-atopic subject GJ29. **a)** Determined by RAST for the allergens; house dust mite (HDM), mixed grass (G), cat (C), *Aspergillus fumigatus* (AF). **b)** Determined by skin-prick test for; *Dermatophagoides* (house dust mite) (HDM), *Phleum pratense* (grass pollen) (G), mugwort (M), three trees (T), silver birch (B), cat (C), dog (D), horse (H), *Aspergillus fumigatus* (AF), *Cladosporium herbarum* (CL), *Alternaria alternata* (A).

The nasal biopsy and PBMC samples were processed exactly as those in section 4.3 (although the cDNA reaction was modified according to a newly available protocol such that even greater efficacy of PCR amplification was enabled (see section 3.2.17 for details). PCR experiments repeatedly yielded V_H-Cε products from the PBMC (*Fig.* 4.4.1). These PCR products appeared to consist of clones exhibiting V_H regions from different classes (data not shown). In contrast, no V_H-Cε PCR products were amplified from the nasal mucosa in any of the repeated experiments (*Fig.* 4.4.1).

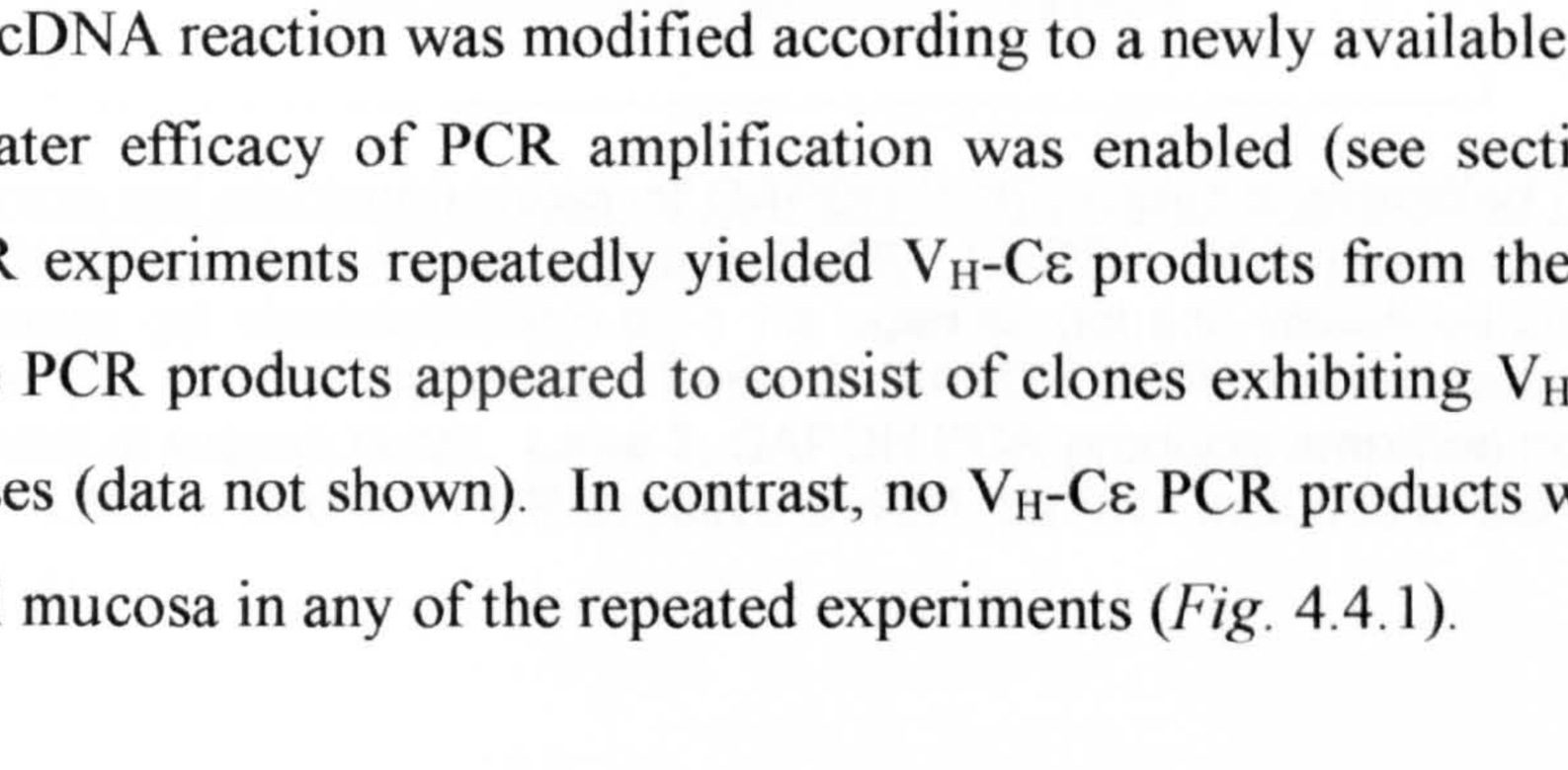


Fig. 4.4.1; Agarose gel electrophoresis of V_H-Cε PCR products amplified from the nasal mucosa and PBMC of non-atopic subject GJ29. V_H-Cε PCR products were subjected to agarose gel electrophoresis on a 1% agarose gel and visualised under UV. **Lane 1**; 100 bp ladder (L), (500bp indicated). **Lane 2**; V_H-Cε PCR products amplified from the nasal mucosa (NM) of subject GJ29. **Lane** 3; V_H-Cε PCR products amplified from PBMC (PB) of subject GJ29. **Lane 4**; V_H-Cε PCR negative control (no RNA in cDNA reaction).

PCR analysis of GAPDH expression in the nasal biopsy and PBMC samples both yielded positive signals, demonstrating that poor sample quality was unlikely to be the cause of the negative V_H-Cε PCR from the nasal biopsy sample (*Fig.* 4.4.2).

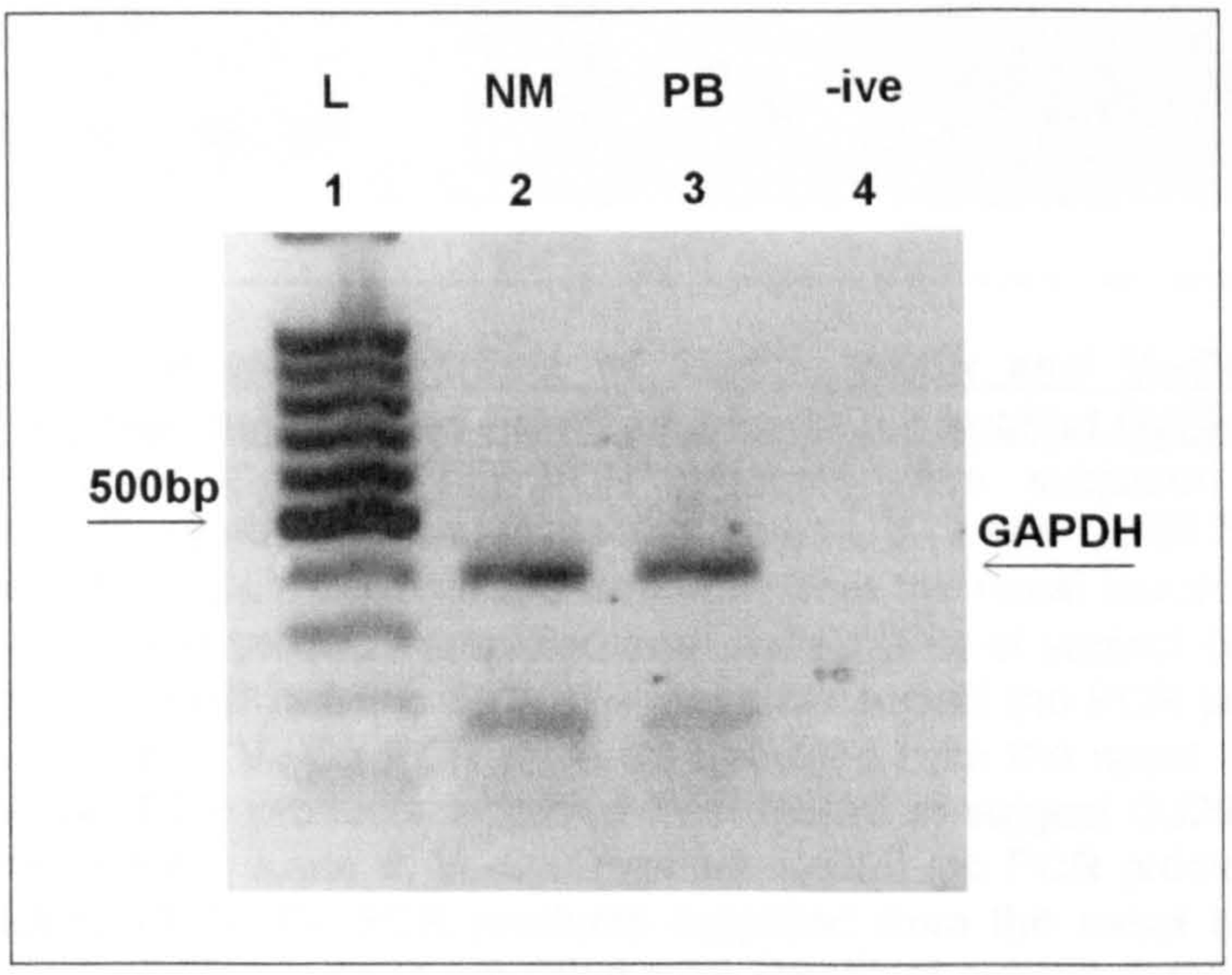


Fig. 4.4.2; Agarose gel electrophoresis of GAPDH PCR products amplified from the nasal mucosa and PBMC of non-atopic subject GJ29. GAPDH PCR products (393 bp) were subjected to agarose gel electrophoresis on a 1% agarose gel and visualised under UV. **Lane 1**; 100 bp ladder (L), (500bp indicated). **Lane 2**; GAPDH PCR products amplified from the nasal mucosa (NM) of subject GJ29. **Lane 3**; GAPDH PCR products amplified from PBMC (PB) of subject GJ29. **Lane 4**; GAPDH PCR negative control (no RNA included in cDNA reaction).

In addition, further PCR experiments yielded products corresponding to V_H-Cμ, V_H-Cα and V_H-Cγ transcripts in both the nasal biopsy and PBMC sample (*Fig.* 4.4.3), suggesting that the inability to PCR amplify V_H-Cε transcripts from the nasal biopsy sample was likely to have resulted from an absence, or very low level, of Cε transcripts in the sample.

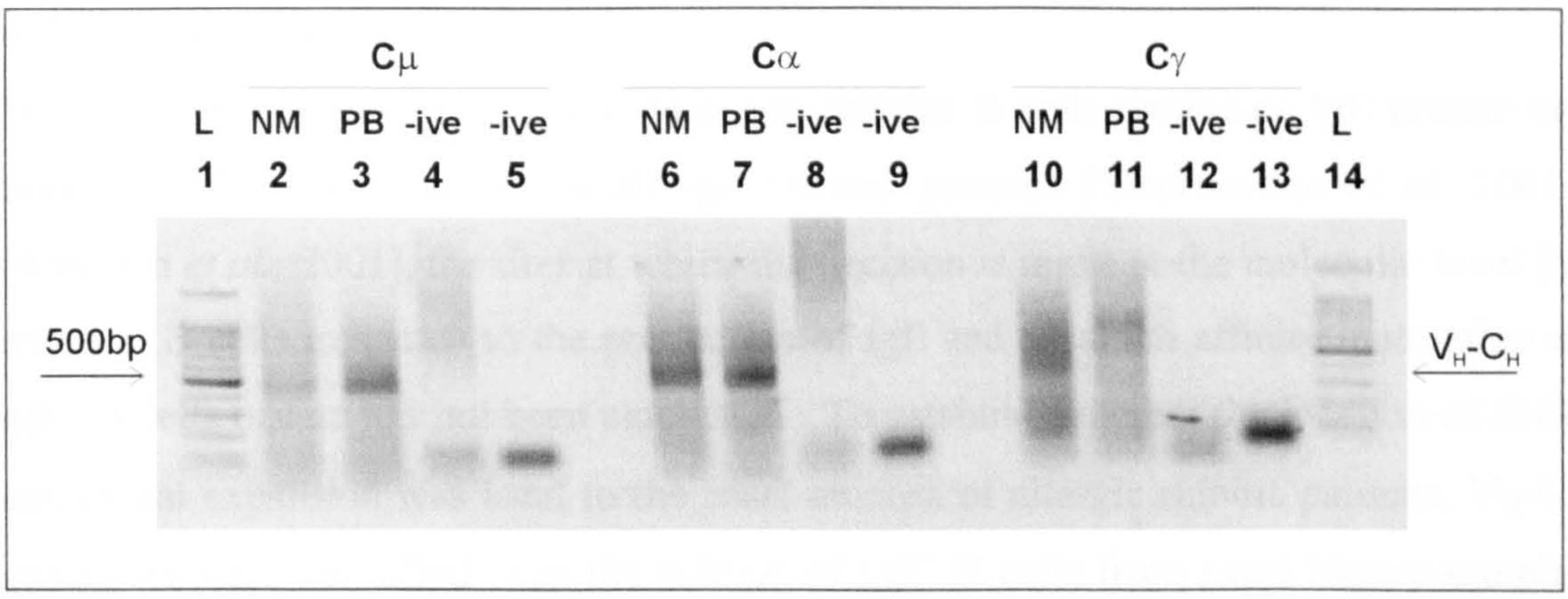


Fig. 4.4.3; Agarose gel electrophoresis of V_H-Cμ, V_H-Cα and V_H-Cγ PCR products amplified from the nasal mucosa and PBMC of non-atopic subject GJ29. V_H-Cμ (450 bp), V_H-Cα (450 bp) and V_H-Cγ (450 bp) PCR products were subjected to agarose gel electrophoresis on a 1% agarose gel and visualised under UV. **Lane 1**; 100 bp ladder (L), (500 bp indicated). **Lane 2**; V_H-Cμ PCR products amplified from the nasal mucosa (NM) of subject GJ29. **Lane 3**; V_H-Cμ PCR products amplified from PBMC (PB) of subject GJ29. **Lane 4**; V_H-Cμ negative control (no cDNA). **Lane 5**; V_H-Cμ negative control (no PCR product carried into the second PCR). **Lane 6**; V_H-Cα PCR products amplified from the nasal mucosa of subject GJ29. **Lane 7**; V_H-Cα PCR products amplified from PBMC of subject GJ29. **Lane 8**; V_H-Cα negative control (no cDNA). **Lane 9**; V_H-Cα negative control (no PCR product carried into the second, PCR). **Lane 10**; V_H-Cγ PCR products amplified from the nasal mucosa of subject GJ29. **Lane 11**; V_H-Cγ PCR products amplified from PBMC of subject GJ29. **Lane 12**; V_H-Cγ negative control (no cDNA). **Lane 13**; V_H-Cγ negative control (no PCR product carried into the second PCR). **Lane 14**; 100 bp ladder.

The possibility that the biopsy contained no amplifiable V_H-Cε transcripts because of sampling (as seen in a small proportion of allergic rhinitis patients with high systemic total IgE) must be acknowledged, although it was not possible to take further nasal biopsies from GJ29 or to recruit similar patients to consolidate these findings. The analysis of GJ29 would seem to suggest that a high total serum IgE value does not result in the random migration of IgE^+ B cells into the nasal mucosa in a non-atopic subject. This data supports the evidence presented in section 4.3 suggesting that the families of clonally related IgE^+ B cells resulted from local SHM and clonal expansion in the nasal mucosa of the allergic rhinitis patients in this study.

4.5 Discussion.

Whilst previous investigators have demonstrated that B cells producing IgE protein are present in the nasal mucosa of allergic rhinitis patients [Smurthwaite *et al*, 2001], [KleinJan *et al.*, 2001], the sites at which the decision is made at the molecular level for mucosal B cells to switch to the production of IgE and at which affinity maturation of IgE^+ B cells occurs has not been elucidated. To establish whether the location of SHM and clonal expansion was local to the nasal mucosa of allergic rhinitis patients, V_H-C_H sequences were amplified from the mRNA of IgE^+ B cells from nasal biopsy samples and compared to those from the peripheral blood of the same allergic rhinitis patients. In this chapter, evidence has been presented that suggests that local SHM and clonal expansion occurs in the nasal mucosa of allergic rhinitis patients and additionally the role that DNA polymerases may play in the misinterpretation of results.

Initial analysis of sequences generated by PCR amplification with *Taq* DNA polymerase from the nasal biopsy and PBMC samples of patient SL5 indicated that the experimental error rate in the system was too high to enable a confident distinction to be made between somatic mutations and experimentally induced errors (despite such data being previously published [Van der Stoep *et al.*, 1993], [Snow *et al.*, 1995], [Snow *et al.*, 1999]). *Pfu* DNA polymerase was shown to incorporate far fewer errors than *Taq*, although the error rate was still such that approximately one in seventeen V_H region sequences were likely to contain an experimental error. Therefore when apparently related B cell clones were *Pfu* amplified in the experiments detailed in this thesis, only relationships confirmed by multiple mutations were acknowledged as genuine.

V_H-Cε sequences isolated from nasal biopsy samples from the cohort of seven allergic rhinitis patients demonstrated reliable evidence of clonally related IgE^+ B cells from the nasal biopsies of two of the patients. V_H region sequence alignment enabled the construction of genealogical trees for three separate families of related IgE^+ B cell clones (two from SO16 and one from AP19, each composed of either two or three isolated IgE^+ B cell clones from the nasal mucosa). The clones from each family displayed clear relatedness, evident from the shared V_H-D-J_H signature region distinct to the clonally related members of each individual family and indicative of the unique V_H-D-J_H recombination that occurred in the progenitor B cell of each family. In addition, each family of B cell clones exhibited both shared and diverse somatic mutations,

indicative both of their shared ancestry and also individual affinity maturation and clonal expansion.

The IgE^+ B cell clones isolated from the nasal mucosa were so closely related and so concentrated that it is probable that they resulted from local SHM and clonal expansion. The less likely alternative would be that each related clone migrated individually from the lymphoid tissue to the same region of the inferior turbinate. This would only seem to be a viable option if the repertoire of IgE^+ B cells in the nasal mucosa was extremely limited, such that if a B cell were to randomly migrate into the mucosa it would be close to a related clone, whereas V_H region sequence analysis in this study showed that a diverse range IgE^+ B cell clones existed in the nasal mucosa.

In contrast to the three families of closely related IgE^+ B cells isolated from the nasal biopsy samples, only one instance of related IgE^+ B cell clones was detected from peripheral blood. Two distantly related IgE^+ B cells were isolated from the PBMC of patient HD17, the only mono-allergic patient and the patient exhibiting the highest total serum IgE. The more distant relationship between the two related PBMC clones was in contrast to the closely related IgE^+ B cell clones isolated from the nasal mucosa. It is possible that such small sampling of the total PBMC pool may not have fully represented the extent of related IgE^+ B cells, which would be distributed throughout the entire volume of peripheral blood.

Instances of identical clones being isolated from both the nasal biopsy and PBMC occurred in three patients (CM10, SO16 and HD17). In each instance the clones were unique and not members of a B cell family that had been isolated, suggesting that they may have resulted from independent migration of identical sister clones into the nasal mucosa and peripheral blood from the lymphoid tissue, or that the clone isolated from the PBMC originated from the nasal mucosa. Alternatively the duplicated sequences may have resulted in each case, from cross-contamination between the nasal biopsy and PBMC samples (at any stage of the experiment), or from the presence of PBMC within the nasal biopsy itself.

In only one instance (SO16) was a clone isolated from the PBMC that was distantly related to a clone isolated from the same patient's nasal biopsy sample. In this instance the clone isolated from the PBMC was related to a family of IgE^+ B cell clones isolated

from the nasal mucosa. It appeared that the PBMC clone may have diverged at an earlier stage than the generation of the family of clones. It is again possible that this peripheral blood B cell clone was generated in the nasal mucosa, but it is also possible that the clone migrated into the peripheral blood from the lymphoid tissue, and that the family of IgE^+ B cells was generated in the nasal mucosa but founded by a sister B cell clone that had also migrated from the lymphoid tissue.

Whilst V_H-Cε sequences were amplified from all of the nasal biopsy and peripheral blood samples in the cohort of seven patients, families of related B cells were only found in the nasal mucosa of SO16 and AP19 and not, as might be expected, from highly allergic patients such as HD17. It is possible that some genuinely related clones were eliminated because of concerns over experimental errors and also that instances of multiple, identical sister clones were overlooked as the difference between multiple identical clones and multiple identical transcripts generated by one clone could not be distinguished in this system and so were assumed to originate from one clone. It would seem likely that if local SHM and clonal expansion occur in the nasal mucosa, that the related cells may be organised in clusters (see chapter 6). It may therefore be the random sampling by a single nasal biopsy from the whole of the inferior turbinate that resulted in the detection of related IgE^+ B cell clones from only two of the seven patients. These clusters of related B cells may be dispersed randomly throughout the inferior turbinate. Multiple biopsies from each patient (a procedure not ethically possible) may be necessary in order to consistently detect B cell clonal families.

While it is unlikely that a family of clonally related IgE^+ B cells would migrate individually from the lymphoid tissue to the same approximate 2.5 mm^3 area of the nasal mucosa, particularly as the V_H-Cε sequences isolated from the nasal mucosa indicated the presence of a diverse rather than extremely limited repertoire of B cells, it was important to eliminate the possibility that a high serum IgE value resulted in the saturation of the nasal mucosa with IgE^+ B cells: PCR analysis of V_H-Cε sequences from the nasal mucosa of a non-atopic subject who exhibited highly elevated serum IgE but no respiratory symptoms was conducted. No V_H-Cε PCR products could be amplified from this nasal biopsy sample, even after repeated attempts, although V_H-Cε PCR products were easily amplified from the peripheral blood.

This research indicates that a high total serum IgE in the absence of allergic rhinitis does not result in the presence of families of related IgE^+ B cell clones in the nasal mucosa, but rather that their presence is inherently linked to the state of allergic rhinitis. Furthermore, in allergic rhinitis patients the existence of families of closely related B cell clones is indicative of successive cycles of local somatic hypermutation and clonal expansion in the nasal mucosa.

Chapter 5

Local class switch recombination in the nasal mucosa of an allergic rhinitis patient.

5.1 Introduction

The data presented in Chapter 4 suggested that local somatic hypermutation and clonal expansion had occurred in the nasal biopsy samples from at least two of the seven allergic rhinitis patients that had been studied. Previous research has suggested by the analysis of Iε and Cε RNA transcripts, that local class switch recombination (CSR) also occurs in the nasal mucosa of allergic rhinitis patients [Durham *et al.*, 1997] and the lung mucosa of allergic asthmatics [Ying *et al.*, 2001]. Further work in the lung implicated the respiratory mucosa as a site of local events when the analysis of V_H transcripts from clonally related B cells was used to obtain evidence for the occurrence of CSR in the lung mucosa of allergic asthmatics [Snow *et al.*, 1999].

As the results presented in chapter 4 suggested that the signals necessary for SHM were present in the allergic nasal mucosa, further work was carried out to determine if any evidence of local CSR could be established in that cohort of patients. This investigation into local CSR was undertaken by the analysis of V_H region sequences based on the methods previously applied to the lung mucosa [Snow *et al.*, 1999]. Research was concentrated on the analysis of sequences from the nasal biopsy samples from which families of related IgE^+ B cell clones had been detected previously. As little evidence of families of related IgE^+ clones in the PBMC was established in chapter 4, with only one instance of two distantly related clones being identified, this area of research was not extended to the peripheral blood.

5.2 Investigation of IgM^+, IgA^+ or IgG^+ B cell clones related to the V_H3 and V_H5 family of IgE^+ B cell clones previously isolated from the nasal biopsy of allergic rhinitis patient SO16.

The PCR protocol utilised to investigate local CSR in the nasal mucosa of allergic rhinitis patients was based on the protocol detailed by previous researchers [Efremov *et al.*, 1993], [Snow *et al.*, 1998] and [Snow *et al.*, 1999], with the addition of a further PCR to validate the results, as described in *Fig.* 5.2.1. The protocol incorporated an initial nested PCR (1 and 2) that specifically amplified all transcripts in which the V_H class of interest (*i.e.* V_H1-V_H7) was present, in conjunction with either Cμ (IgM), Cα (IgA) or Cγ (IgG), each as a separate reaction. A subsequent, highly specific semi-nested PCR (reaction 3) was then employed to further amplify from PCR 2 any cDNA

expressing the clonal signature region unique to the particular B cell clonal family of interest. If the semi-nested PCR 3 successfully demonstrated the presence of either Cμ, Cα or Cγ transcripts in conjunction with the signature region specific for the IgE family of clones, further semi-nested PCRs, each using a sample from PCR 2 were used to determine the exact V_H region sequence (PCR 4) and also, in this study, to confirm the validity of the full length of sequence, from the highly mutated CDR1 of a B cell clone through to the C_H region (PCR 5).

The rational behind this experimental approach was that the presence of B cell clones related to a family of IgE^+ B cells, but that expressed IgM, IgA or IgG in the same nasal biopsy sample implied that local CSR had occurred, resulting in the occurrence of such geographically close, related B cell clones of different isotypes.

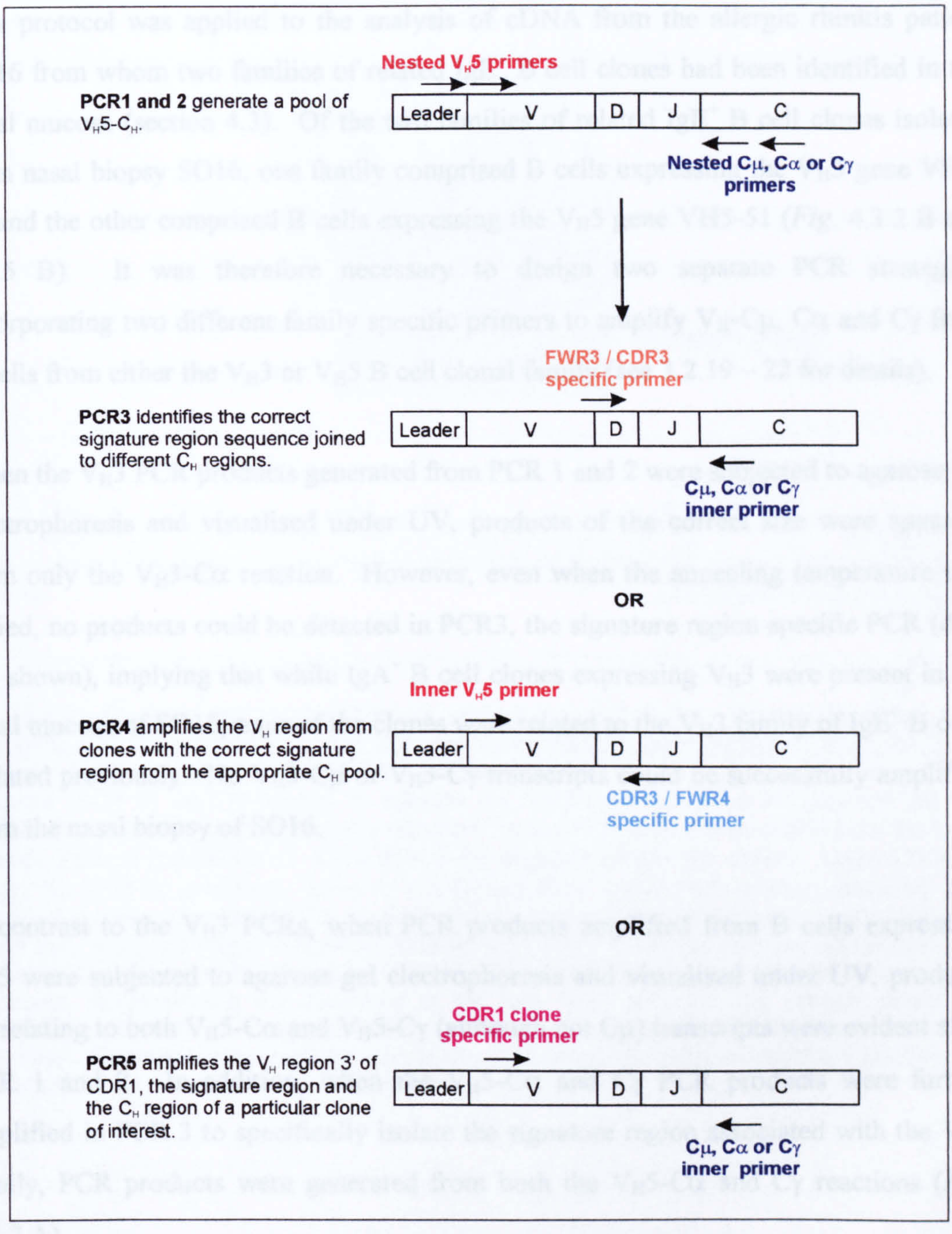


Fig. 5.2.1; Diagrammatic representation of the PCR protocol for the amplification of V_H-Cμ, V_H-Cα and V_H-Cγ sequences from specific B cell clones. To amplify V_H-C_H sequences from B cells of a particular clonal origin, an initial nested PCR was undertaken in which (in the case of a B cell expressing a V_H5 gene) V_H5-Cμ, V_H5-Cα, and V_H5-Cγ sequences were each amplified separately by nested PCR 1 and 2. A semi-nested PCR 3 was then used to amplify the specific signature region of the required clone from a sample taken from PCR 2, utilising the inner Cμ, Cα, or Cγ primers in conjunction with the appropriate primer (unique to each clonal family) that bridged the V_H-D junction of the signature region. If the appropriate signature region was isolated from PCR 3 a further sample of PCR 2 was used in the semi-nested PCR 4 to amplify from the inner V_H5 primer to the specific D-J_H junction of the signature region. To ensure that the fragments of sequence amplified in PCR 3 and 4 formed one complete transcript, a sample of the appropriate PCR 2 was used in a semi-nested PCR 5, to amplify from the uniquely mutated CDR1 of the particular B cell clone to the inner Cμ, Cα, or Cγ primer. The PCR products generated by PCR 2 were gel purified before use in subsequent reactions. PCR products from PCR 3, 4 and 5 were cloned and sequenced. PCR 1 and 2 each utilised 30 cycles of amplification and PCR 3, 4 and 5, 20 cycles of amplification. Based on a protocol detailed by [Efremov *et al.*, 1993], [Snow *et al.*, 1998], [Snow *et al.*, 1999].

This protocol was applied to the analysis of cDNA from the allergic rhinitis patient SO16 from whom two families of related IgE^+ B cell clones had been identified in the nasal mucosa (section 4.3). Of the two families of related IgE^+ B cell clones isolated from nasal biopsy SO16, one family comprised B cells expressing the V_H3 gene VH3-30 and the other comprised B cells expressing the V_H5 gene VH5-51 (*Fig.* 4.3.2 B and 4.3.5 B). It was therefore necessary to design two separate PCR strategies, incorporating two different family specific primers to amplify V_H-Cμ, Cα and Cγ from B cells from either the V_H3 or V_H5 B cell clonal family (see 3.2.19 – 22 for details).

When the V_H3 PCR products generated from PCR 1 and 2 were subjected to agarose gel electrophoresis and visualised under UV, products of the correct size were apparent from only the V_H3-Cα reaction. However, even when the annealing temperature was varied, no products could be detected in PCR3, the signature region specific PCR (data not shown), implying that while IgA^+ B cell clones expressing V_H3 were present in the nasal mucosa of SO16, none of the clones were related to the V_H3 family of IgE^+ B cells isolated previously. No V_H3-Cμ or V_H3-Cγ transcripts could be successfully amplified from the nasal biopsy of SO16.

In contrast to the V_H3 PCRs, when PCR products amplified from B cells expressing V_H5 were subjected to agarose gel electrophoresis and visualised under UV, products correlating to both V_H5-Cα and V_H5-Cγ (although not Cμ) transcripts were evident after PCR 1 and 2. In addition, when the V_H5-Cα and Cγ PCR products were further amplified in PCR 3 to specifically isolate the signature region associated with the V_H5 family, PCR products were generated from both the V_H5-Cα and Cγ reactions (*Fig.* 5.2.2 A).

When the V_H5-Cα and Cγ PCR 3 products were cloned and sequenced, the V_H5-Cγ sequences did not contain the correct signature region. In contrast, the signature region sequence generated from the V_H5-Cα PCR (*Fig.* 5.2.2 B) was identical to that of the related family of IgE^+ B clones isolated previously from the nasal mucosa of SO16. Instead of expressing Cε however, the signature region was joined to Cα2. This indicated the presence of at least one B cell clone that originated from the same progenitor cell as the component members of the V_H5 family of IgE^+ B cell clones, but which had undergone CSR to IgA_2.

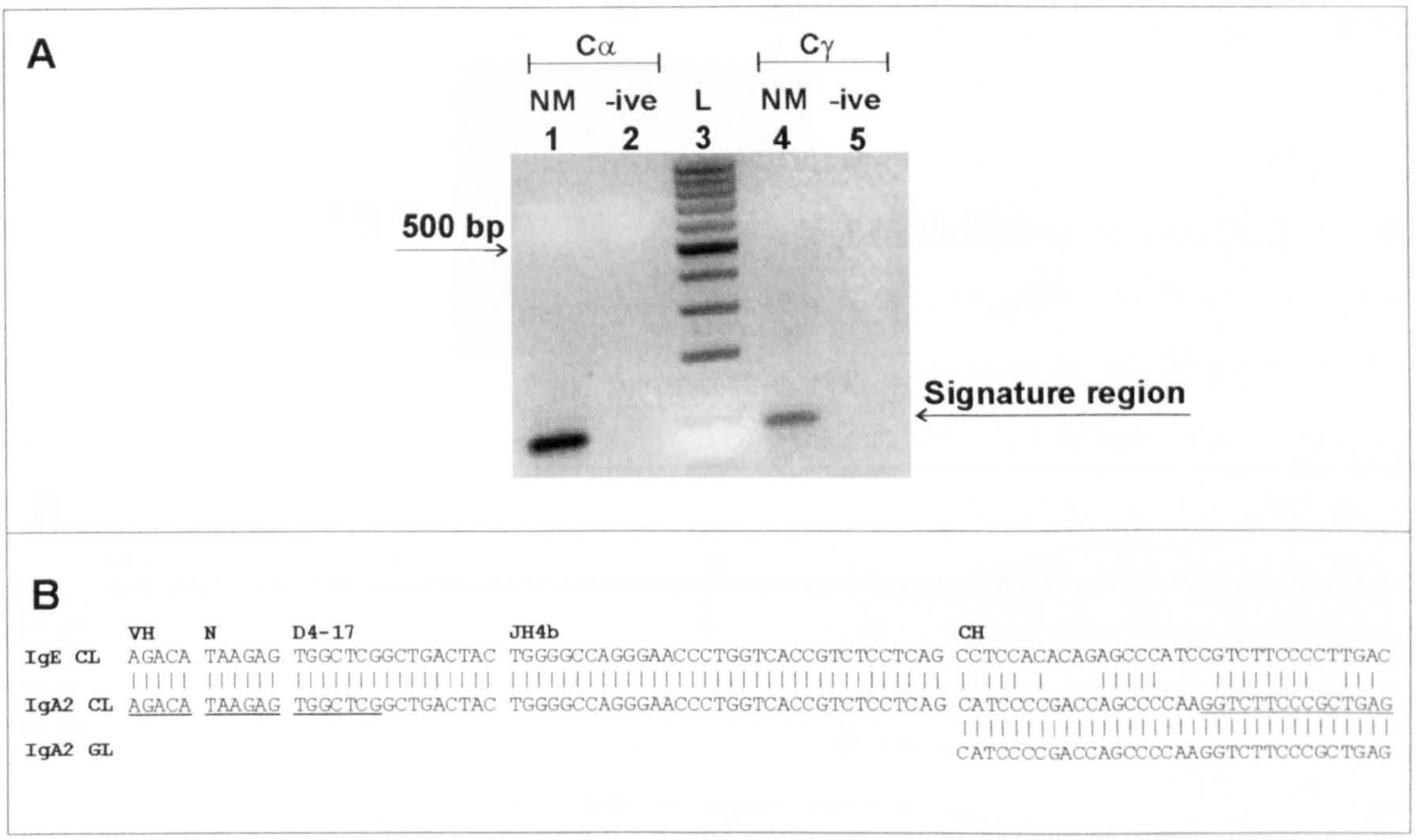
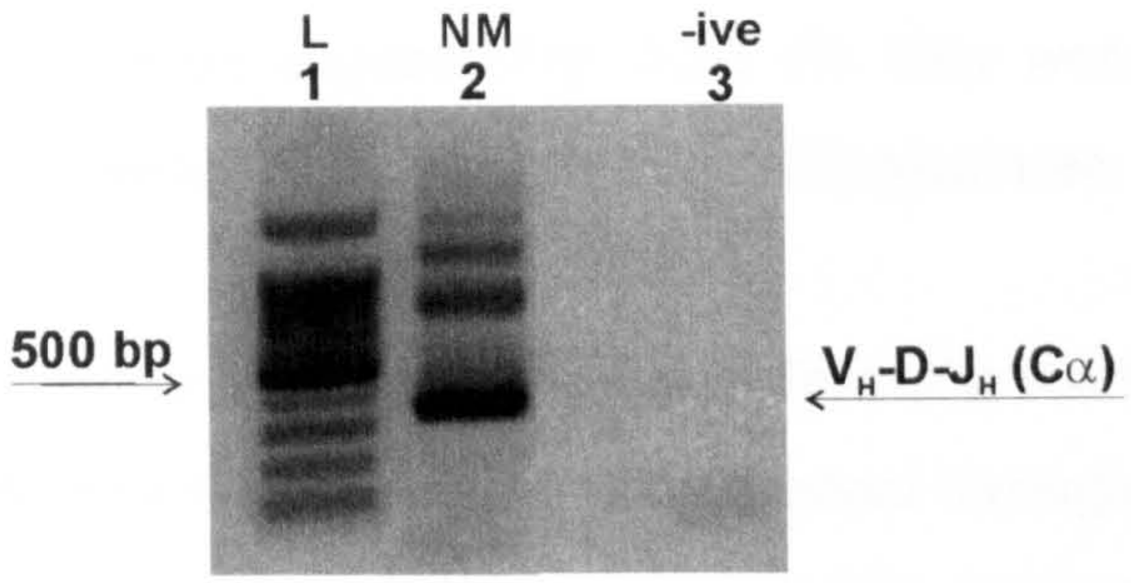


Fig. 5.2.2; Agarose gel electrophoresis of V_H5-Cα and V_H5-Cγ signature region PCR products and the sequence of the IgA_2 signature region, both amplified from the nasal mucosa of patient SO16. **A)** V_H5-Cα (95 bp) and V_H5-Cγ (94 bp) signature region PCR products (PCR 3) were subjected to agarose gel electrophoresis on a 2% agarose gel and visualised under UV. **Lane 1**; V_H5-Cα signature region PCR products amplified from the nasal mucosa (NM) of patient SO16. **Lane 2**; V_H5-Cα negative control (no cDNA). **Lane 3**; 100 bp ladder (L), (500 bp indicated). **Lane 4**; V_H5-Cγ signature region PCR products amplified from the nasal mucosa of patient SO16. **Lane 5**; V_H5-Cγ negative control (no cDNA). **B)** Alignment of the IgA_2 signature region sequence isolated from PCR 3, aligned with the signature region sequence exhibited by the related IgE^+ B cell clones and also aligned with the IgA_2 germline sequence, demonstrating the homology of the IgA_2 clone with the IgE^+ clones across the signature region but homology with IgA_2 in the constant region. CL represents a clonal sequence. GL represents a germline sequence. Homology between nucleotides is represented by a vertical line. Primer regions are underlined.

In order to establish the relationship of the IgA_2 clone(s) with the V_H5 IgE^+ B cell clonal family, PCR 4 was carried out to amplify the V_H region and enable analysis of the somatic mutations. The resultant PCR products were subjected to agarose gel electrophoresis (5.2.3 A), cloned and fifteen sequences analysed. Five of the fifteen clones clearly shared the correct V_H-D junction. In addition, analysis of the V_H region sequences from the IgA clones demonstrated that while some mutations were common to the IgA clones and some were the same as those in the related IgE^+ B cell clones, all exhibited additional unique mutations throughout the length of the V_H region (*Fig.* 5.2.3 B).



B

```
                                                                                                  -----CDR1------
          VH5-51                    10                  20                  30                  40
GL        GAGGTGCAGCTGGTGCAGTCTGGAGCAGAGGTGAAAAAGCCCGGGGAGTCTCTGAAGATCTCCTGTAAGGGTTCTGGATACAGCTTTACCAGCTACTGGATCGGCTGGGTGCGCCAGATGC
IgE C11   ..........................................C.........a.........................A....G...C.....CC.....................
IgE C14   ..........................................C.........a.........................t.A....G...C.....CC.....................
IgE C8    ..........................................C.........a...........A.........t.A....G...C.....CC.....................
IgA C1    ..............................C.............C.....................................t.AG...G...C...tGCC.....t.............
IgA C2    ..............................C.........g..a.....................................a.........c.............................
IgA C5    ........................g.................g.....................................C.....A.............CG.........C.............
IgA C14   ........................G...a.............C.....................................t.AG...G...C...tGCC.....t.............
IgA C17   ..........................................T.g.....................................AC..........T.....G..C.C.........C.............

                                                  ----------CDR2----------
                                  50                  60                  70                  80
GL        CCGGGAAAGGCCTGGAGTGGATGGGGATCATCTATCCTGGTGACTCTGATACCAGATACAGCCCGTCCTTCCAAGGCCAGGTCACCATCTCAGCCGACAAGTCCATCAGCACCGCCTACCTG
IgE C11   .......g.................................A.........A....C.....................C.........A........T.............A.............
IgE C14   .......g.................................A.........A....C.....................C.........A........T.............A.............
IgE C8    .......g.................................A.........A....C...t.................C.........A........T.............A.............
IgA C1    ....a.....................................................t.C.....................C.........A........T.............A..........t..
IgA C2    ..........................G...C..T....A.........A.......C....t.....tC.....................Tg............GA.............
IgA C5    ..........................................................G...C.....................................................A..........t..
IgA C14   ..............................a.......A.........GT......tG..G.....................................................................
IgA C17   ..........................C.......c..cAAc.....................................T...........c..............At...A.........

                                                                  90                  N       D4-17               JH4b
GL        CAGTGGAGCAGCCTGAAGGCCTCGGACACCGCCATGTATTACTGTGCGAGACA ------ TGACTACGGTGACTAC TGGGGCCAG
IgE C11   ............G....G.....................T..................... tAAGAG ..G..CG.C....... .........
IgE C14   ............G.....................T..................... tAAGAG ..G..CG.C....... .........
IgE C8    ............G.....................T..................... tAAGAG ..G..CG.C....... .........
IgA C1    ..C.........G.....................A.....t.................. tAAGAG ..G..CG.C....... .........
IgA C2    ..................................T..................... tAAGAG ..G..CG.C....... .........
IgA C5    ..C.........G.....................A.....t.................. tAAGAG ..G..CG.C....... .........
IgA C14   ..C...C.....................G......C..................... tAAGAG ..G..CG.C....... .........
IgA C17   ............G.....................T..................... tAAGAG ..G..CG.C....... .........
```

Fig. 5.2.3; Agarose gel electrophoresis of V_H-D-J_H PCR products from IgA$^+$ B cell clones and the sequence alignment of those IgA sequences with the related IgE$^+$ B cell clones previously amplified from the nasal mucosa of patient SO16. **A)** V_H-D-J_H PCR products from IgA$^+$ B cells (327 bp) (PCR 4) were subjected to agarose gel electrophoresis on a 2% agarose gel and visualised under UV. **Lane 1**; 100 bp ladder (L), (500 bp indicated). **Lane 2**; V_H-D-J_H PCR products amplified IgA$^+$ B cells from the nasal mucosa (NM) of patient SO16. **Lane 3**; V_H-D-J_H negative control (no cDNA). **B)** Alignment of V_H-D-J_H sequences (expressing the genes VH5-51, D4-17 and JH4b) amplified from related IgA$^+$ and IgE$^+$ B cell clones isolated from the nasal mucosa of patient SO16. GL represents the closest germline gene homology as determined by VBase. N denotes non-templated nucleotide insertions. The primer regions used for amplification of the IgA clones are underlined and no mutations in the primer regions are included. Homology to GL is represented by a dot, mutations from GL are in uppercase (if a replacement amino acid mutation) or lowercase (if a silent amino acid mutation). CDR regions are detailed.

While the IgA$^+$ and IgE$^+$ B cell clones were clearly related, it was difficult to construct a meaningful genealogical tree since the range of mutations was so diverse. Furthermore, there were instances where identical mutations occurred in what must have been different branches of the clonal family, making it difficult to reliably establish the exact

position of some of the IgA clones within the family. Consequently, although both the IgA and IgE sequences were aligned (*Fig.* 5.2.3 B), they were not used to construct what may have been an erroneously structured genealogical tree.

When the alignment of the V_H region sequences from the related IgA and IgE clones was studied, evidence of antigen selection was observed throughout the family of B cell clones; an average of 5.6% of the FWR was mutated in contrast to 12.7% of the CDR. Moreover, when the replacement / silent (R/S) amino acid mutation ratio of all the mutations accumulated within the family was compared in the CDRs as opposed to the FWRs, the CDR R/S ratio was 4.2, whereas in the FWR the R/S ratio was 2.6. This high R/S ratio in the CDRs is indicative of conventional antigen selection across the region [Shlomchik *et al.*, 1987].

Although a copy of the correct signature region, identical to that expressed by the IgE^+ V_H5 family of related B cells, had been amplified from an IgA_2^+ B cell in PCR 3, the possibility could not be excluded that the V_H region sequences detailed in 5.2.3 B, that had been assumed to be from IgA^+ B cells, may have instead resulted from IgE sequences non-specifically amplified by the IgA primer set (as PCR 4 did not extend to amplify the C_H region). It was also possible that while the signature region had been amplified from an IgA_2 cell, some or all of the V_H sequences may have originated from an IgA_1 B cell(s). The amplification of the full V_H-D-J_H-Cα sequence was not possible in PCR 4 because of the inability to amplify from the inner V_H5 primer through to the Cα region, whilst still maintaining the specificity for sequences from the particular B cell clonal family. Attempts to amplify from the inner V_H5 primer to the J_H-C_H junction were unsuccessful because of the largely conserved nature of the 3' region of the J_H genes (data not shown).

Previous researchers had assumed that the V_H region sequences amplified in PCR 4 would have originated from the same isotype as the signature region [Efremov *et al.*, 1993], [Snow *et al.*, 1998], [Snow *et al.*, 1999]. To be doubly sure, a novel PCR5 was designed in this study for control purposes. The (supposedly IgA^+) B cell clone C1 was chosen at random and a primer designed that was specific to its (considerably mutated) CDR1. In PCR 5 the V_H5-D-J_H-Cα sequence of clone C1 3' to CDR1 was amplified.

PCR 5 successfully amplified a sequence identical in the V_H-D-J_H sequence previously amplified from the clone C1, although in addition the V_H region was joined to Cα2 (*Fig.* 5.2.4). (An additional base mutation in the D region was evident, although this may have accumulated experimentally as the signature region and all somatic mutations were otherwise identical). This CDR1-D-J_H-Cα sequence confirmed the validity of the previous results, suggesting that at least one of the IgA^+ sequences was derived from an IgA_2 B cell. Time constraints prevented analysis of the other four IgA clones by PCR5.

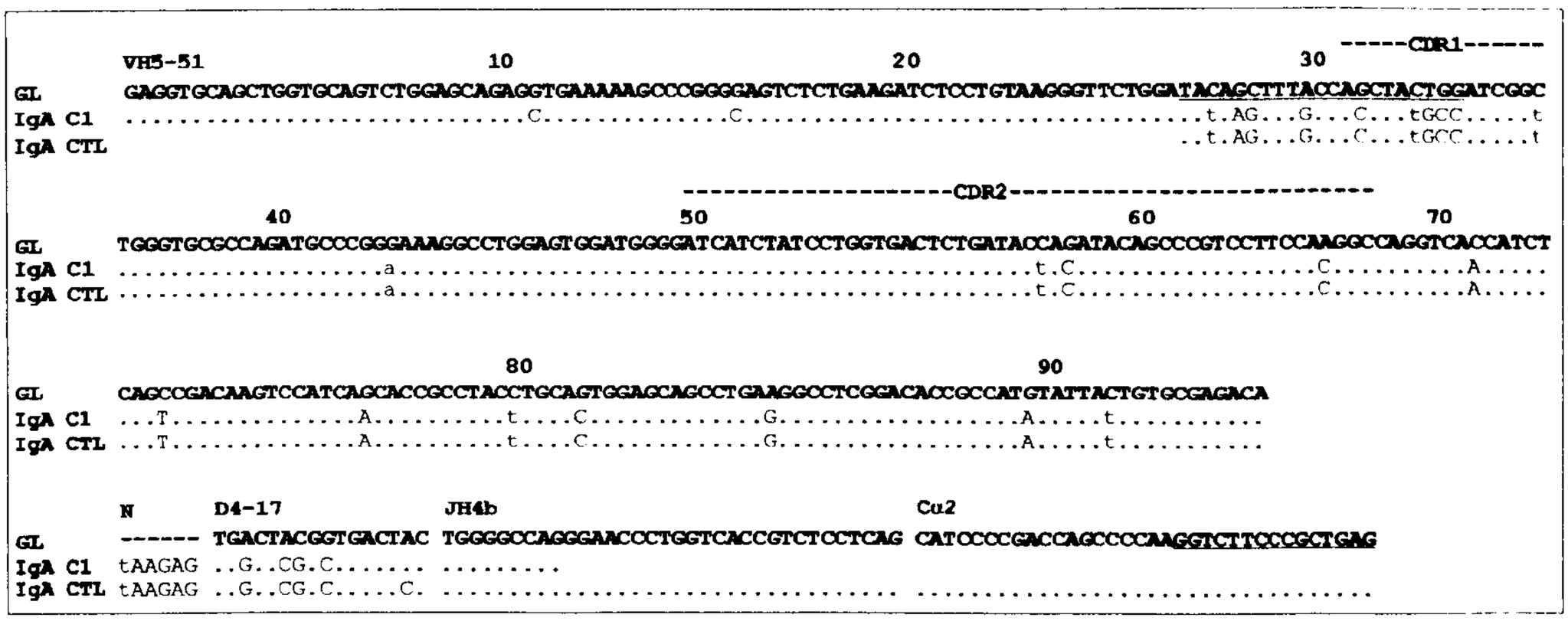


Fig. 5.2.4; Sequence alignment of the V_H-D-J_H sequence amplified from clone C1 in PCR4 with the CDR1-D-J_H amplified in PCR5, confirming that C1 was indeed an IgA_2 clone. Both sequences were isolated from the nasal mucosa of patient SO16. PCR 5 specifically amplified from the mutated CDR1 of the sequence from the B cell clone C1, through to the IgA_2 constant region (IgA CTL), confirming that the sequence isolated from clone C1 in PCR 4 (IgA C1) was indeed from an IgA_2^+ B cell. Both sequences expressed the genes VH5-51, D4-17 and JH4b and were isolated from the nasal mucosa of patient SO16. GL represents the closest germline gene homology as determined by VBase. N region constitutes non-templated nucleotide insertions. Primer regions for the amplification of the control sequence are underlined and no mutations in these regions were included. Homology to GL is represented by a dot, mutations from GL are in upper case (if a replacement amino acid mutation) or lower case (if a silent amino acid mutation). CDR regions are detailed.

5.3 Investigation of IgM^+, IgA^+ or IgG^+ B cell clones related to the V_H5 family of IgE^+ B cell clones previously isolated from the nasal biopsy of allergic rhinitis patient AP19.

As sequences from IgA^+ B cell clones had been isolated from the nasal biopsy of SO16, that were clearly related both to each other and also to the V_H5 IgE^+ family of B cell clones from the same nasal biopsy, it seemed likely that related clones of an isotype other than IgE might be detected in nasal biopsy AP19. A family of related V_H5 IgE^+ B

cell clones had been previously isolated from AP19 (*Fig.* 4.3.2 D and 4.3.5 C). PCR 1 and 2 therefore targeted amplification of cDNA from V_H5-Cμ, Cα and Cγ transcripts in the same manner as for SO16. When the PCR 2 products were subjected to agarose gel electrophoresis and visualised under UV, DNA bands indicative of V_H5-Cμ, Cα and Cγ clones were observed.

PCR 3 was employed as previously using a primer specific for the particular V_H-D junction of the V_H5 family from AP19 in order to amplify the correct signature region, joined to C_H. In contrast to SO16, no appropriate signature region sequences were isolated, for while PCR products, particularly from Cγ, were evident upon agarose gel electrophoresis, when cloned and sequenced it was clear that all of the products had been non-specifically amplified. No further progress could be made, suggesting that while V_H5 clones expressing IgM, IgA and IgG were present in the nasal biopsy AP19, none appeared to be related to the V_H5 family of clonally related IgE^+ B cells. Time constraints did however mean that experiments were focused on the investigation of SO16 and these experiments were not repeated multiple times. It may therefore have been that the clones could not be easily detected, rather than that they were absent from the sample.

5.4 Investigation of IgM^+, IgA^+ or IgG^+ B cell clones related to the V_H3 family of IgE^+ B cell clones previously isolated from the nasal biopsy of allergic rhinitis patient CM10.

As the experimental work described in section 4.3 progressed, it became evident that certain B cell clonal families should be regarded as unreliable as a consequence of the experimental error rate. Initially however, an attempt was made to investigate whether B cell clones expressing isotypes other than IgE, related to the family of related IgE^+ B cell clones from nasal biopsy CM10 could be amplified. The apparent family of related clones isolated from the nasal mucosa of CM10 was small, comprising two V_H3 B cell clones that differed by just one mutation (*Fig.* 4.3.2 A). The primers used in PCR 1 and 2 were targeted to amplify cDNA from V_H3-Cμ, V_H3-Cα and V_H3-Cγ transcripts. These initial PCRs successfully amplified V_H3-Cα and V_H3-Cγ cDNA (data not shown). However, sequence analysis demonstrated that the signature region products amplified in the subsequent PCR 3 were generated non-specifically, regardless of the

annealing temperature used in PCR 3. No evidence could therefore be provided to suggest that local CSR had occurred in the nasal biopsy sample from allergic rhinitis patient CM10.

5.5 Discussion

A greater body of research has been directed towards the study of local class switch recombination than towards the study of local somatic hypermutation because of its appeal as a potential therapeutic target for the prevention of the local allergic response. Examples of previous research include *in situ* hybridisation studies of nasal biopsy samples which demonstrated that while the number of B cells in the nasal mucosa did not increase after stimulation with grass pollen allergen, the proportion that expressed Iε and Cε did, indicative of the stimulation of epsilon germline transcription; the first step in CSR to IgE [Durham *et al.*, 1997]. However, this approach did not conclusively demonstrate that the molecular process of CSR had occurred locally and it did not indicate which isotypes CSR was occurring between, or enable analysis of V_H region sequences from the relevant B cell clones.

An alternative approach, in which V_H region sequences were analysed from an asthmatic patient, was used to demonstrate local CSR, in the allergic lung mucosa [Snow *et al.* 1999]; V_H region sequences from clonally related IgM^+, IgG^+ and IgE^+ B cells were identified, suggesting that it was likely that local CSR had occurred (rather than the alternative; that the related clones had been subjected to CSR in the lymphoid tissue and then migrated to exactly the same site of biopsy). While this approach did not conclusively demonstrate local CSR it again suggested its likelihood.

Conclusive evidence of local CSR has very recently been demonstrated by the presence of switch circle transcripts in the nasal mucosa of allergic rhinitis patients (a project undertaken in the Gould group) [Takhar *et al.*, manuscript in preparation] and supported by evidence that DNA switch circles (generated by both Cμ to Cε and additionally Cγ to Cε switches) are stimulated in the nasal mucosa as a consequence of *ex vivo* allergen challenge [Cameron *et al.*, 2003]. It was however of interest to apply the technique of V_H region sequence analysis for the first time to nasal biopsy samples and to investigate local CSR in samples from allergic rhinitis patients that had previously been analysed

(section 4.3) to determine if the results correlated with those observed in the allergic lung mucosa [Ying *et al.*, 2001], [Snow *et al.*, 1999] and in the allergic nasal mucosa [Durham *et al.*, 1997], [Cameron *et al.*, 2003], [Takhar *et al.*, manuscript in preparation].

The data presented in section 5.2 suggested that there was no evidence of local CSR in the nasal biopsy samples from the allergic rhinitis patients CM10 or AP19. With hindsight this was perhaps not surprising in CM10, as the analysis of the PCR error rate later suggested that the family of related IgE^+ B cell clones was unlikely to be genuine. Perhaps more surprising was the lack of evidence to support local CSR in the nasal biopsy from AP19 as previous analysis of the IgE^+ clones had suggested on the basis of reliable data the presence of a family of at least three related IgE^+ B cell clones in the nasal biopsy (thus suggesting the microenvironment might be suitable for local CSR).

It is possible that no related clones from alternative isotypes were detected because the family of IgE^+ B cells was founded in the nasal mucosa by a B cell that had already switched to IgE outside of the nasal mucosa (as discussed in section 4.5 there would seem to be no reason why such events in the lymphoid tissue would not occur in addition to those in the nasal mucosa). Alternatively, if CSR had occurred locally in AP19, related clones of different isotypes may have been outside of the biopsied area. It must also be acknowledged that the PCR strategy may simply have failed to successfully isolate clones that were present, particularly as while non-specific products were isolated from PCR 3, no specific positive control could be applied to the reaction.

In contrast to the results from CM10 and AP19, while no evidence of non-IgE^+ B cells related to the V_H3 family of B cell clones from the nasal biopsy of SO16 was established, there was clear evidence suggestive of local CSR associated with the V_H5 family of IgE^+ B cell clones from SO16; a signature region sequence demonstrating the presence of an IgA_2 clone related to the family of V_H5 IgE^+ B cell clones was isolated. Furthermore, when the V_H region sequences from the IgA clones were analysed it was evident that at least a further four related IgA clones existed in the nasal biopsy sample. Concern over the specificity of the PCR reactions was alleviated by evidence that the sequence of a clone that had been assumed to originate from an IgA^+ B cell was indeed joined to a $C\alpha2$ constant region.

The IgA clones expressed some mutations that were identical to those observed in the IgE^+ clones and some that differed. The different mutations were evident throughout the sequence and not, for example, only at the 5' or 3' end which might have otherwise suggested the isolation of experimental artefacts. It was also of interest that while the construction of a genealogical tree displaying the exact relationships between the clones could not be reliably constructed, there did appear to be evidence of antigen selection, both in that the percentage mutation and the R/S ratio was higher in the CDR than the FWR (indicative of greater antigen selection of replacement mutations across the CDR).

The somatic mutations observed in the IgA clones further supported the data presented in chapter 4 in suggesting that local SHM and clonal expansion had occurred locally in this allergic rhinitis nasal biopsy sample. Furthermore, it appeared that local CSR had occurred locally, rather than the unlikely event of clones in the lymphoid tissue, switching and then each migrating individually to exactly the same site of biopsy in the nasal mucosa. Although the analysis of the clonal repertoire of the whole inferior turbinate of SO16 (if this could have been surgically removed) may have strengthened this suggestion (as it is possible to argue that a huge proportion of the B cell population may have been related and therefore that random migration might result in close proximity to a sister clone), previous sequence analysis (section 4.3) did not suggest that the diversity of B cell clones in the nasal mucosa of SO16 was that restricted.

While it may not perhaps have been surprising to see evidence of a switch from IgM, or particularly IgG to IgE, related IgE and IgA B cell clones were of particular interest, IgA being regarded as the most important antibody isotype in the healthy respiratory tract [Lamm, 1997]. It is however possible that local CSR may not have been between IgE and IgA, but rather that separate incidences of local CSR from related IgM or IgG B cell clones to both IgE and also IgA may have occurred (although no V_H-Cμ sequences were detected).

The predominant stimulant for CSR to either IgA_1 or IgA_2 has been demonstrated to be TGFβ [Islam *et al.*, 1991], although IL-4 and CD40 ligand have also been demonstrated to stimulate the same switch in a lymphoma B cell line [Cerutti *et al.*, 1998]. Some researchers have suggested however that for CSR to occur to different isotypes, a

certain number of cell divisions must have occurred *e.g.* five for CSR to IgE in the mouse [Hasbold *et al.*, 1998]. If the local CSR had taken place between IgE and IgA, it is possible that in a family of clones such as this, clearly highly specific and maturing in affinity in response to an antigen, stimulation to clonally expand may have resulted in a high number of cell divisions favouring a CSR event from IgE to IgA_2.

The repertoire of clones evident in the nasal biopsy samples of CM10, SO16 and AP19 are also worthy of comment. The broadly specific PCR 1 and 2 provided evidence of IgE, IgA and IgG clones in the nasal mucosa. However while the IgM PCR had been successfully used to amplify IgM transcripts in previous experiments, only in nasal biopsy AP19 was evidence of IgM (V_H5) PCR products detected. It is therefore possible that while IgM was clearly evident in the non-atopic GJ29 nasal biopsy (section 4.4), in the allergic nasal mucosa there may be less IgM^+ B cells, although a more detailed study would need to be carried out for this observation to be reliable.

The results presented in this chapter suggest that all of the major antibody isotypes appear to be expressed in the allergic nasal mucosa, although it is possible either that (V_H3 and V_H5) IgM^+ cells are present to a lesser extent or that this PCR is less efficient. In addition, related B cell clones expressing antibody isotypes other than IgE do not appear to be an automatic consequence of the presence of a family of related IgE^+ B cell clones in the allergic nasal mucosa. However, clear evidence has been presented to suggest that in one of the three reliable families of related IgE^+ B cell clones described in section 4.3, at least five related IgA^+ B cell clones exhibiting a variety of shared and conserved somatic mutations across the V_H regions were also present. While this data strengthens the evidence for local SHM and clonal expansion proposed in chapter 4, importantly it additionally suggests that local CSR occurs in the nasal mucosa of allergic rhinitis patients.

Chapter 6

The distribution of IgE$^+$ B cells in the nasal mucosa of allergic rhinitis patients.

6.1 Introduction.

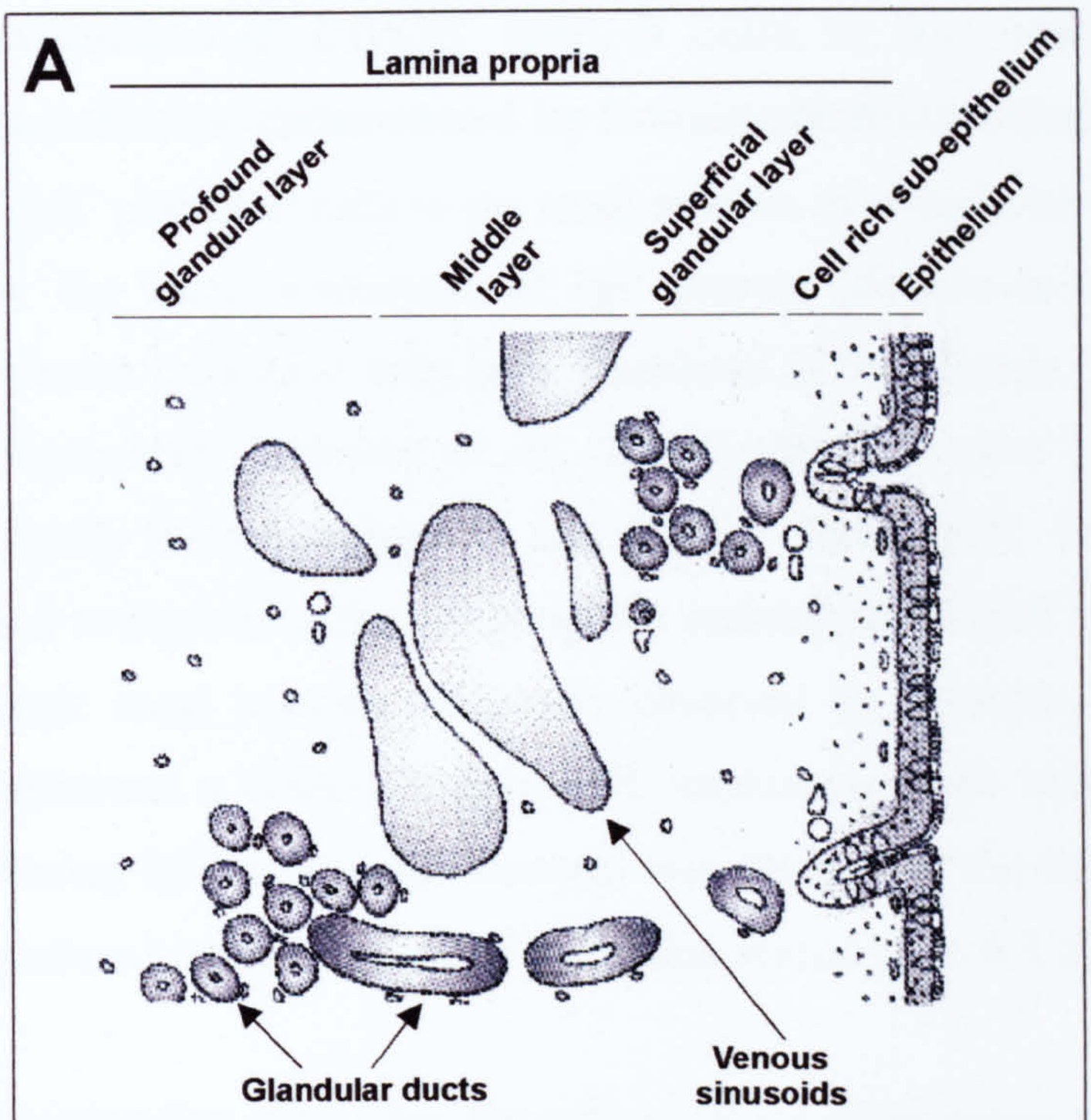
It was evident from the results obtained in chapters 4 and 5 that it was important to learn more about the distribution of IgE$^+$ B cells in the nasal mucosa of allergic rhinitis patients, both in terms of the types and also the geographical distribution of the B cells. If, for example, related B cells were present in distinct clusters, this might suggest that sampling was indeed responsible for the detection of families of related B cell clones from only some patients. To this end, two different experimental approaches were utilised:

Firstly, immunohistochemical (IHC) staining of frozen sections from nasal biopsies was undertaken. It was intended that this would provide data on the geographical distribution of the IgE$^+$ B cells within the nasal mucosa and also the type of B cells present *i.e.* CD19$^+$ B cell, or terminally differentiated CD138$^+$ plasma cell. Initially, IHC experiments were designed to enable the quick identification of IgE$^+$ B cells for subsequent single cell microdissection and RT-PCR analysis of V_H-Cε sequences isolated from those single B cells. This would have additionally enabled sequence data to be generated from either a single or a particular group of IgE$^+$ B cells. Unfortunately, due to technical problems (see below), microdissection of the B cells was not attempted, although the preliminary IHC results alone generated interesting results.

The second experimental approach consisted of the sequence analysis of RT-PCR amplified V_H-Cε transcripts from adjacent pieces of the nasal mucosa, either two halves of the same biopsy, two adjacent biopsies, or two adjacent pieces of tissue excised from a whole inferior turbinate. It was intended that data generated in this manner would provide an insight into how wide the distribution of related IgE$^+$ B cell clones was across the allergic nasal mucosa

6.2 The anatomy of the nasal mucosa.

In the following sections of this chapter the distribution of IgE$^+$ B cells in the nasal mucosa of allergic rhinitis patients is considered. Sections 6.3 and 6.4 describe IHC experiments in which it is informative for the anatomy of the nasal mucosa to be understood. In *Fig.* 6.2.1 the features of the nasal mucosa, including the epithelium, lamina propria and glandular ducts are identified at both a diagrammatic (A) and microscopic (B) level. The position of the cell rich sub-epithelial layer is of particular relevance.



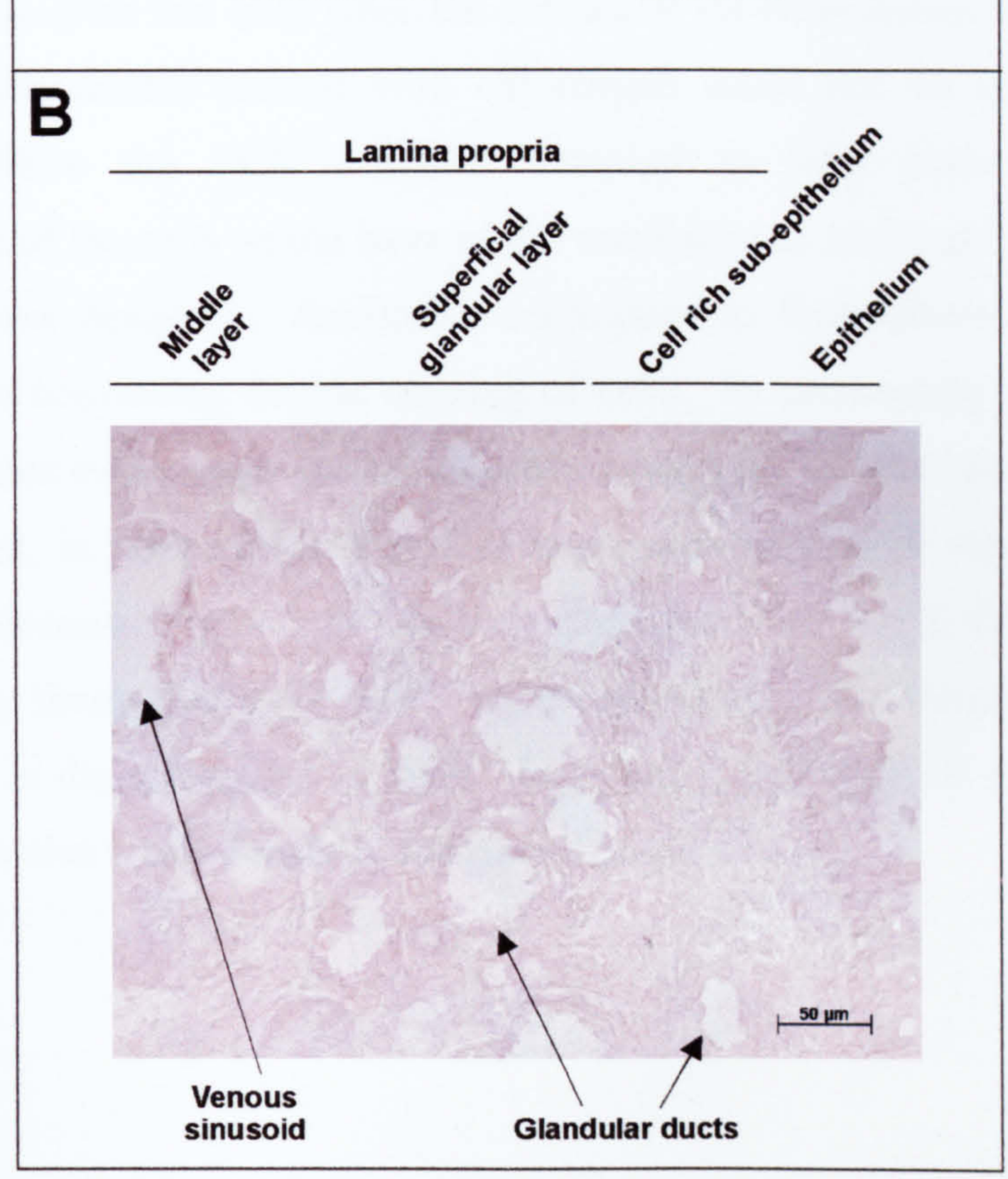


Fig. 6.2.1; The anatomy of the nasal mucosa on the inferior turbinate. **A)** A diagrammatic representation of the nasal mucosa [adapted from Mygind *et al.*, 1996] illustrating the different layers of the nasal mucosa, the glandular ducts and the venous sinusoids. **B)** A typical unstained, 6 µm frozen nasal biopsy section from an allergic rhinitis patient, in which the different regions of the nasal mucosa are delineated as accurately as possible and examples of glandular ducts and venous sinusoids are detailed.

6.3 The distribution of CD138$^+$ IgE$^+$ B cells in the nasal mucosa of allergic rhinitis patients, determined by immunohistochemistry.

The presence of IgE$^+$ plasma B cells in the nasal mucosa of allergic rhinitis patients has been implied by the local production of IgE protein [Smurthwaite *et al.*, 2001]. However, IgE$^+$ plasma cells have only been visualised in the allergic nasal mucosa by one group of researchers [KleinJan *et al.*, 2000], although other researchers have attempted to identify them [Durham *et al.*, 1997]. Initial IHC experiments were therefore designed to reproduce the chromogenic staining of CD138$^+$ IgE$^+$ (plasma) B cells in the allergic nasal mucosa previously observed by KleinJan *et al.* In these preliminary experiments a double stained cell, expressing both IgE and CD138 (a plasma cell producing IgE in the nasal mucosa) was detected in the nasal mucosa of an allergic rhinitis patient biopsied outside of the pollen season (*Fig.* 6.3.1).

It was evident however that even when the colours of the chromogens were varied (data not shown), cells double stained with chromogen could not be un-controversially identified. While the PCR analysis, intended to take place subsequent to microdissection of the cells would have added credibility to such results, an alternative IHC protocol was designed. Antibodies conjugated to fluorophores enabled both a faster and more convincing double staining of cells. In preliminary experiments this protocol generated evidence of another double stained IgE$^+$ CD138$^+$ plasma B cell from the same patient, in which distinct surface expression of CD138 was combined with strong IgE expression (*Fig.* 6.3.2). In both *Figs.* 6.3.1 and 6.3.2, CD138$^+$ plasma B cells, including those that were IgE$^+$, were individually distributed throughout the lamina propria in the sub-epithelial layer. In contrast, IgE$^+$ CD138$^-$ cells, likely to be mast cells, were also found deeper in the lamina propria.

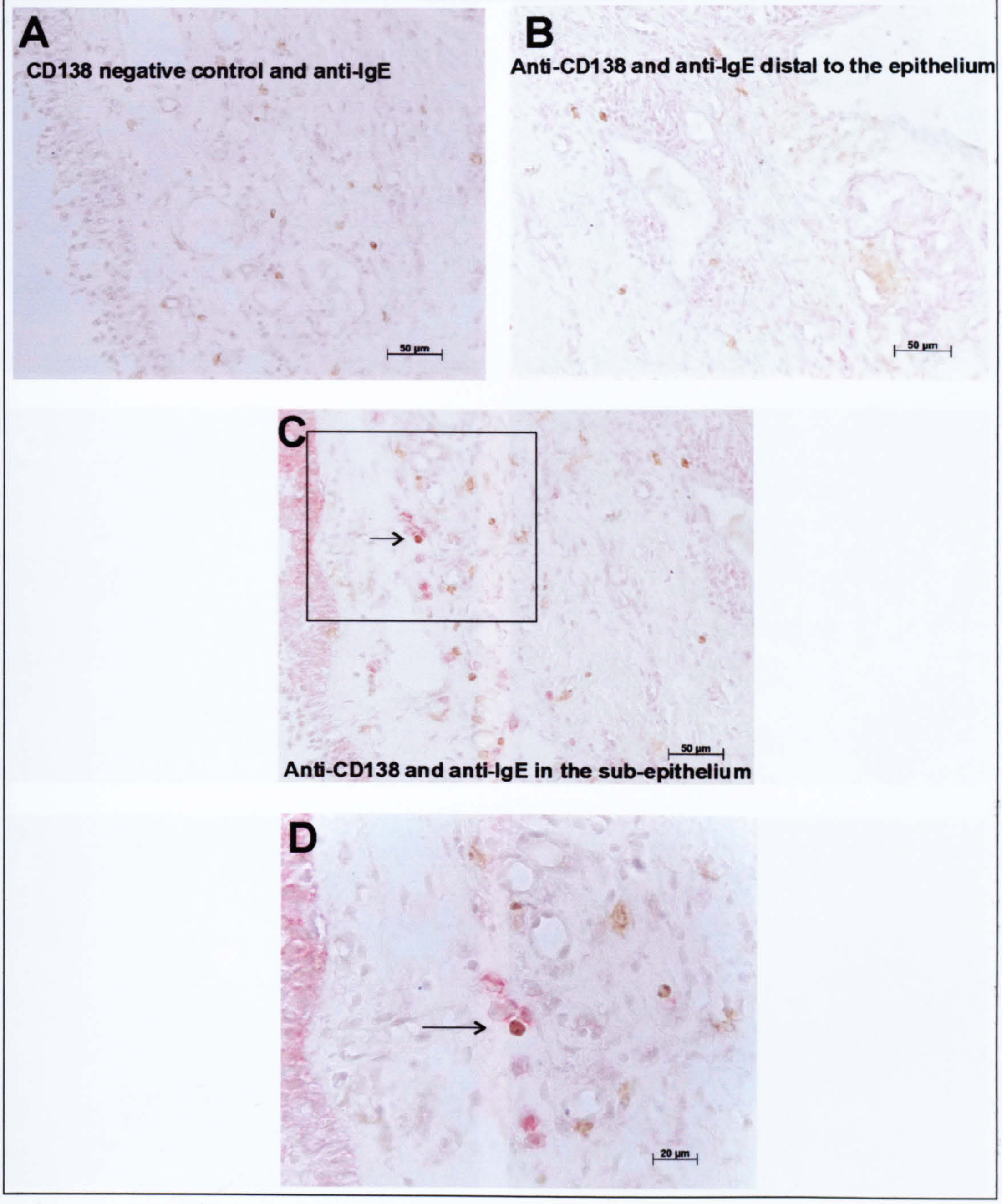
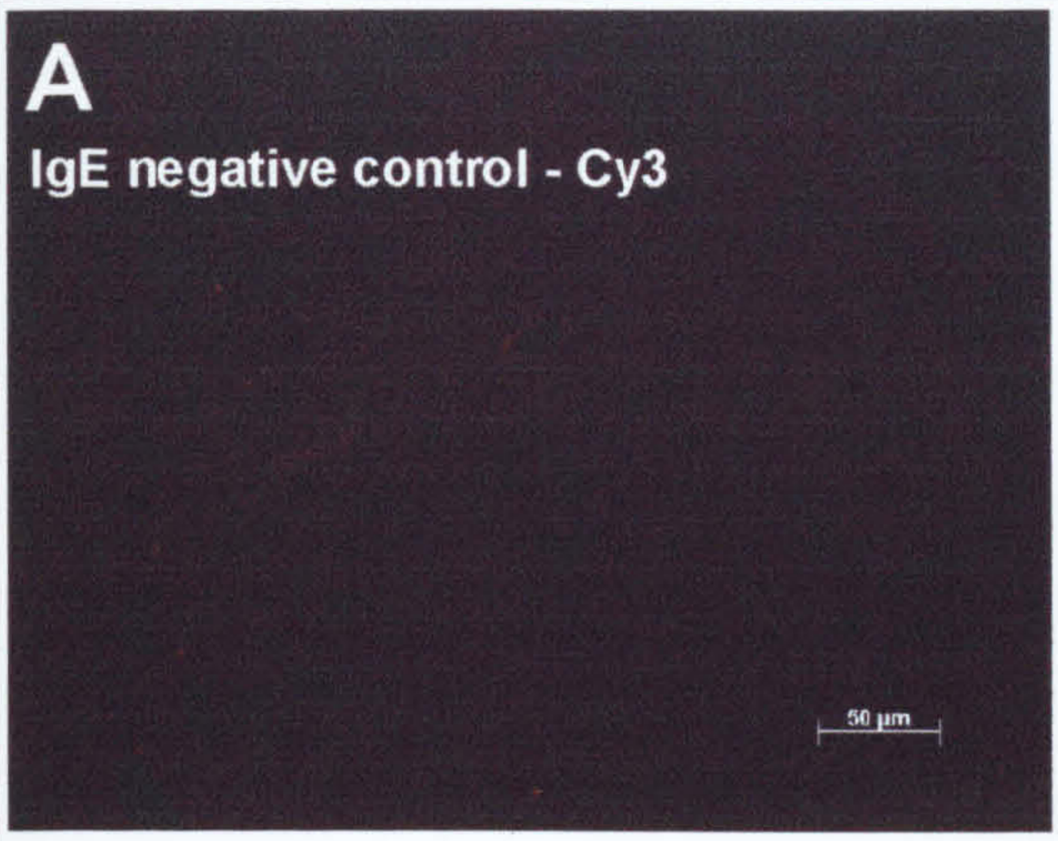
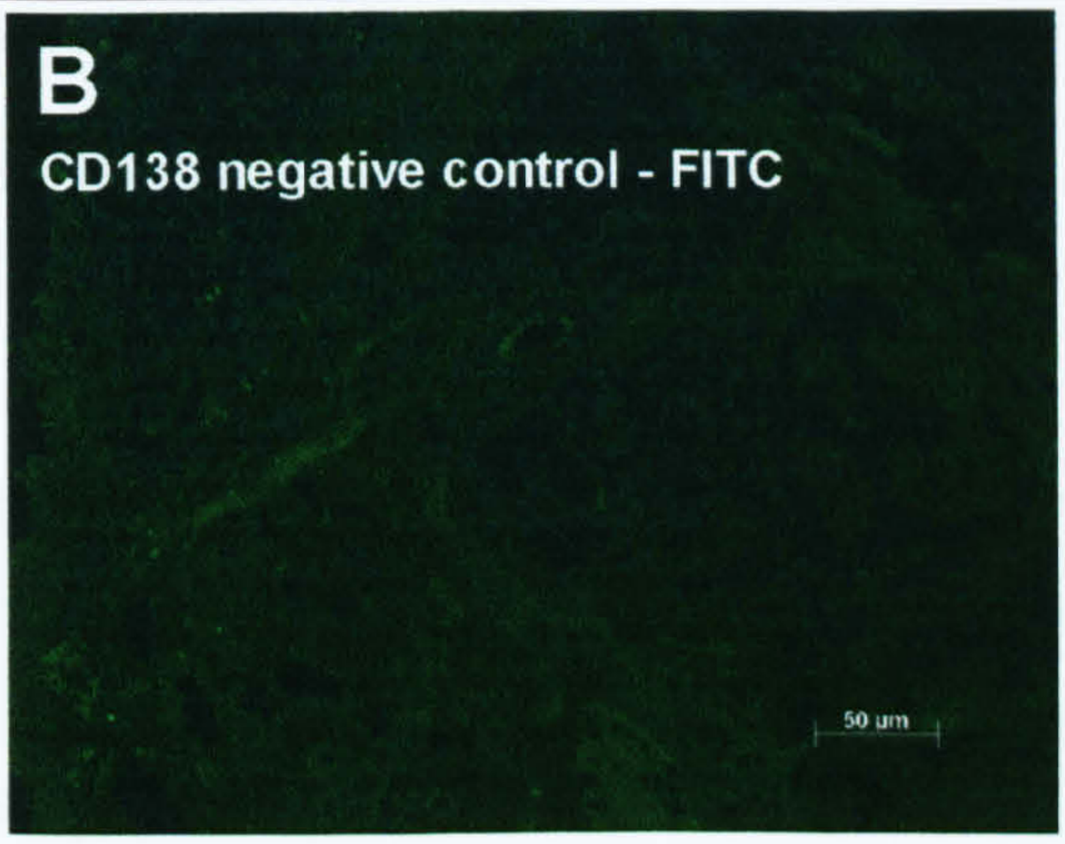
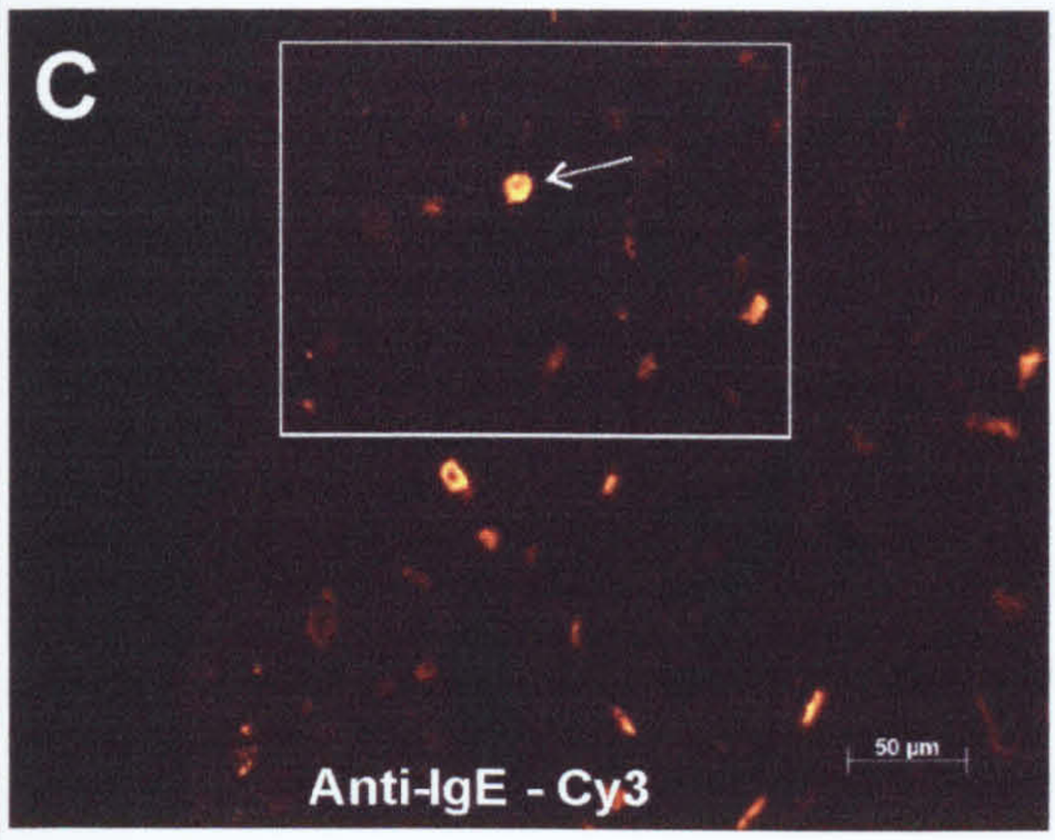


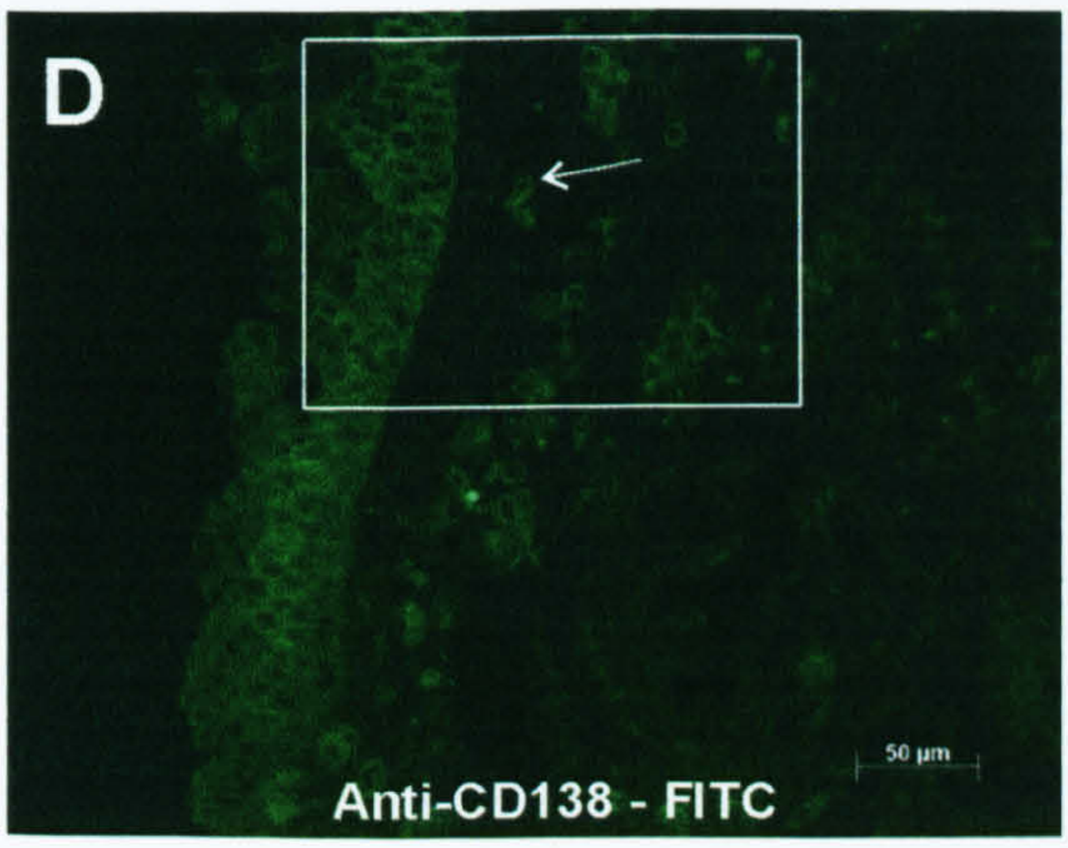
Fig. 6.3.1; Immunohistochemical chromogen staining of IgE and CD138 using 6 µm frozen nasal biopsy sections prefixed in paraformaldehyde from an allergic rhinitis patient outside of the grass pollen season. Fuschin chromogenic substrate was used to immunohistochemically stain for the plasma B (and epithelial) cell marker CD138 (pink). DAB chromogenic substrate was used to immunohistochemically stain for IgE (brown). In A), C) and D) the epithelium is towards the left of the section. In B) the epithelium is not shown but the orientation of the section is the same. For details of the anatomy of the nose please refer to *Fig.* 6.2.1. The sections were incubated with A) mouse MOPC21 (as an isotype control for CD138) and goat anti-human IgE-biotin antibodies and in B), C) and D) mouse anti-human CD138 and goat anti-human IgE-biotin antibodies (see section 3.2.38 for full experimental details). In B) a lack of CD138$^+$ cells was evident in the area of the section distal to the epithelium, although IgE$^+$ cells were present. In C) a double stained IgE$^+$ CD138$^+$ cell, a plasma cell producing IgE is indicated by an arrow. In D) this double stained IgE$^+$ CD138$^+$ cell is shown at a higher magnification. Scale bars representing 50 µm are shown in A) B) and C) and 20 µm in D). No isotype control for the goat anti-human IgE-biotin antibody was available at the time of this experiment, although in a repeat experiment goat-IgG-biotin isotype control antibody gave negative results as expected (data not shown, as only single positive cells were identified). In addition, further experiments confirmed that sections incubated without antibody were also negative (data not shown).

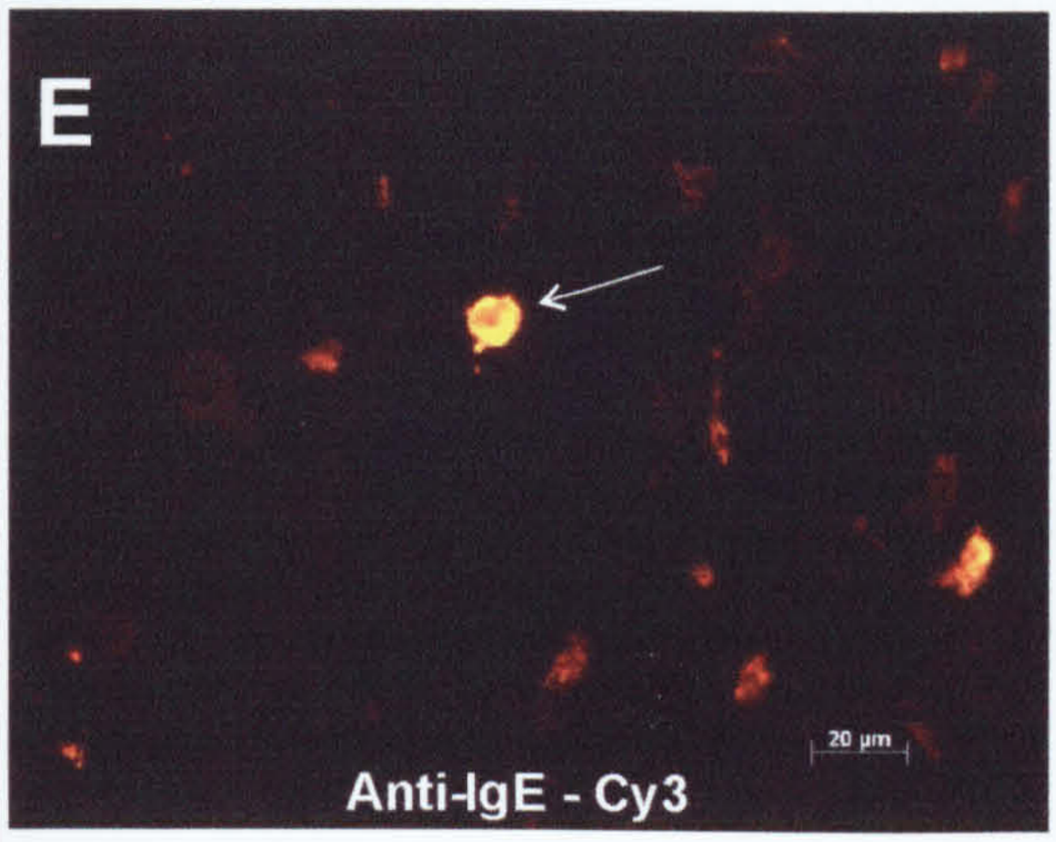
Fig. 6.3.2: Immunohistochemical fluorescent staining of IgE and CD138 using 6 μm frozen nasal biopsy sections prefixed in paraformaldehyde from an allergic rhinitis patient outside of the grass pollen season. The sections presented here originated from the same biopsy as those in *Fig.* 6.3.1. The fluorophore FITC (green) was used to immunohistochemically stain for the plasma B (and epithelial) cell marker CD138. The fluorophore Cy3 (orange) was used to immunohistochemically stain for IgE. A) and B), C) and D), and E) and F) are pairs of images taken from the same section. A), C) and E) were images visualised in the Cy3 spectrum and B), D) and F) in the FITC spectrum. In all sections the epithelium is towards the left of the section. For details of the anatomy of the nose please refer to *Fig.* 6.2.1. The sections were incubated with A) and B) mouse MOPC21 (an isotype control for CD138) and goat IgG-biotin (an isotype control for IgE). In C) and D) mouse anti-human CD138 and goat anti-human IgE mouse antibodies (see section 3.2.39 for full experimental details), a double stained $CD138^{+}$ IgE^{+} cell in the sub-epithelium indicated by an arrow. In E) and F) this double stained cell is shown at higher magnification. In G) the images from E) and F) are merged. Scale bars representing 50μm are shown in A), B), C) and D) and 20μm in E), F) and G). A full complement of control sections were included in this experiment, all of which yielded negative results as expected (data not shown).

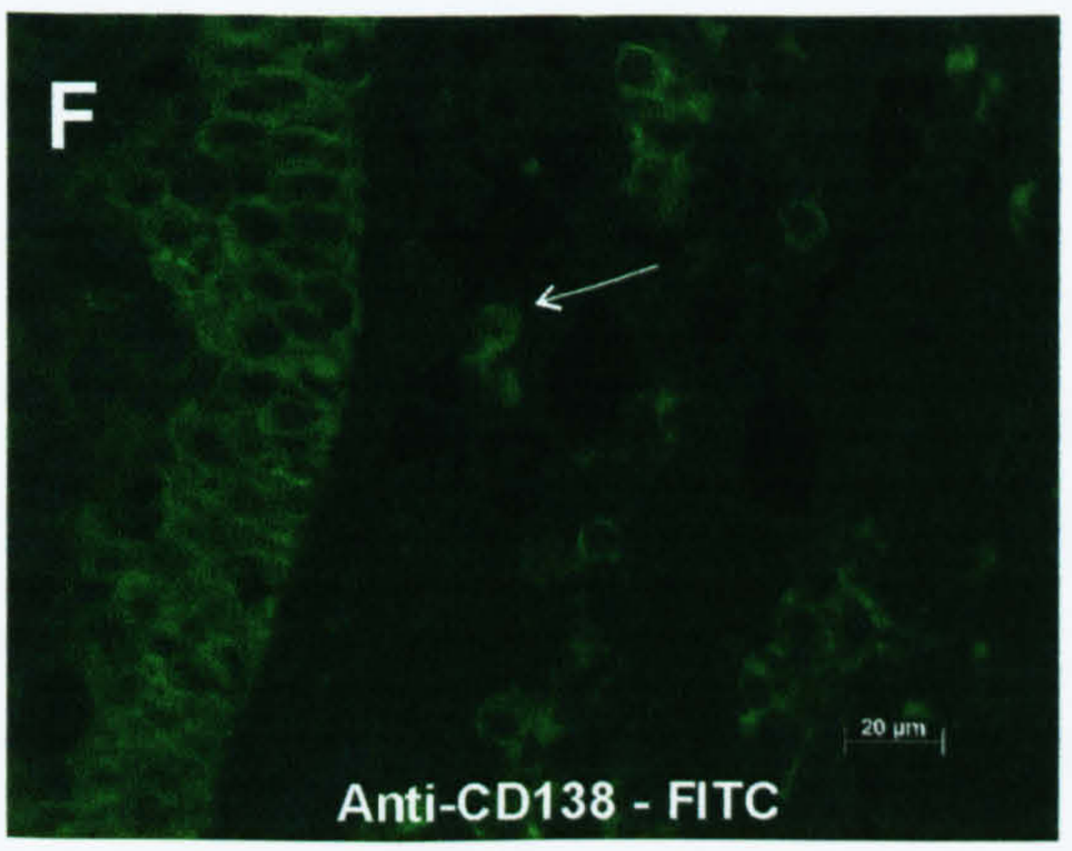

A
IgE negative control - Cy3
50 µm

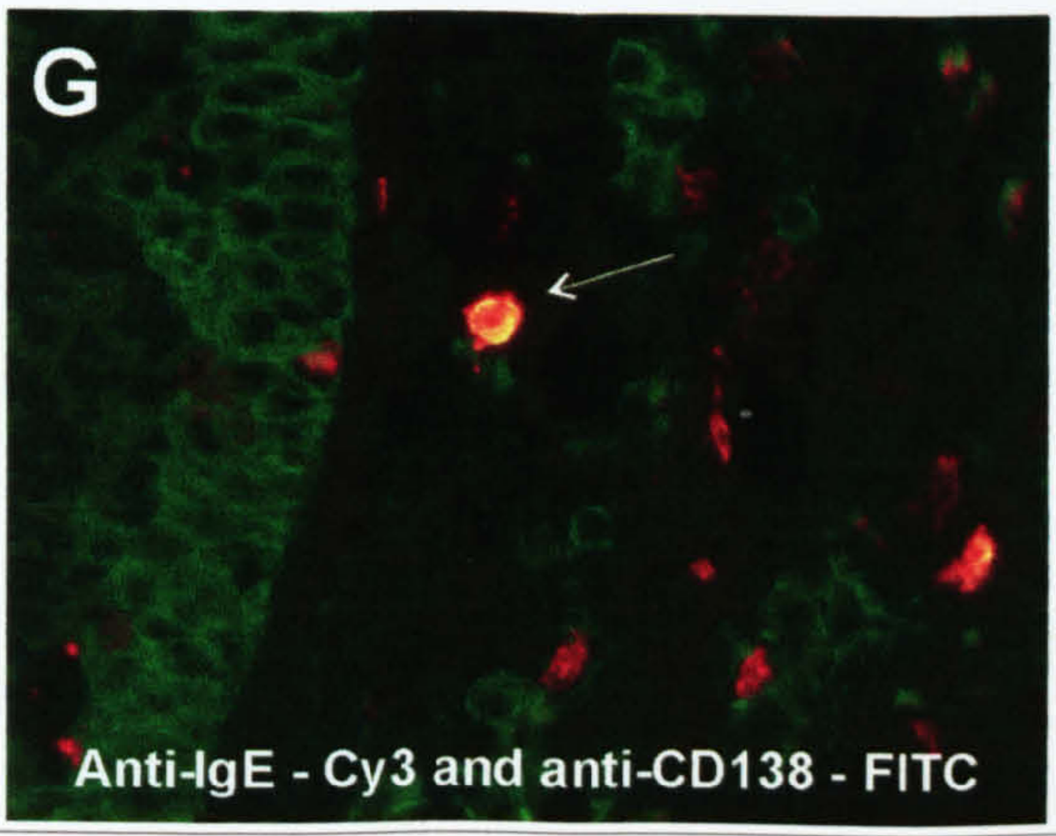

B
CD138 negative control - FITC
50 µm


C
Anti-IgE - Cy3
50 µm


D
Anti-CD138 - FITC
50 µm


E
Anti-IgE - Cy3
20 µm


F
Anti-CD138 - FITC
20 µm


G
Anti-IgE - Cy3 and anti-CD138 - FITC

6.4 The distribution of CD19$^+$ B cells in the nasal mucosa of allergic rhinitis patients, determined by immunohistochemistry.

While the V_H-Cε transcripts from the nasal mucosa, analysed in chapters 4 and 5, may have originated from plasma B cells (particularly as their quantity of V_H-C_H transcripts would be greatest), plasma cells are no longer subject to CSR and SHM. This implies that sequences from related B cell clones would only have been likely to have originated from plasma cells, if a family of related CD19$^+$ B cell clones had undergone terminal differentiation, but remained in close proximity. While only a small number of sections were analysed, IgE$^+$ plasma cells did not appear to behave in this manner according to the preliminary data presented in *Figs*. 6.3.1 and 6.3.2. It was therefore with particular interest that IHC to determine the distribution of CD19$^+$ (non-plasma) B cells in the nasal mucosa was carried out.

Initial IHC staining was undertaken using a chromogenic technique (*Fig*. 6.4.1). These results were then confirmed using fluorescently labelled antibodies (*Fig*. 6.4.2). Unfortunately, while the sections depicted in both *Figs*. 6.4.1 and 6.4.2 were meant to have originated from an allergic rhinitis patient, the hospital were unable to confirm that the nasal biopsy had not mistakenly originated from a normal patient.

The preliminary work did however suggest that while the CD19$^+$ B cells were located in the cell rich sub-epithelium of the lamina propria, they were not as tightly associated with the sub-epithelium as the plasma cells had appeared to be. Importantly, in contrast to the plasma cells, the CD19$^+$ B cells were evident both individually and also as distinct clusters. Problems with the cross-reactivity if the anti-human IgE and anti-human CD19 antibodies unfortunately meant that there was insufficient time to carry out the intended double staining of CD19$^+$ IgE$^+$ B cells in the allergic nasal mucosa during this project.

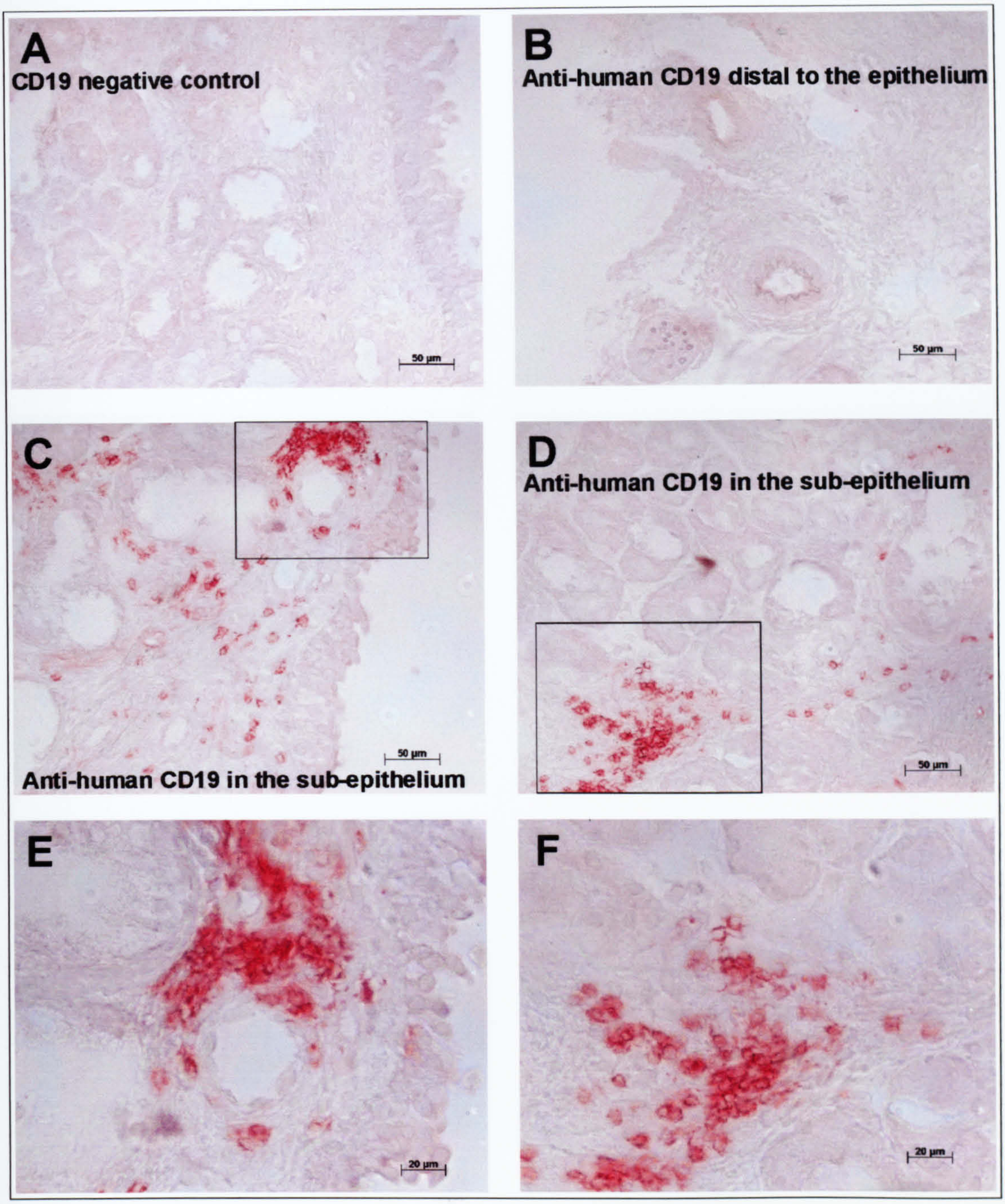


Fig. 6.4.1; Immunohistochemical chromogen staining of CD19 using 6 µm acetone fixed frozen nasal biopsy sections. The allergic status of the subject was not clear. Fuschin chromogenic substrate was used to immunohistochemically stain for the B cell marker CD19. In A), C) and E) the epithelium is towards the right of the section. In B), D) and F) the epithelium is not shown but the orientation of the sections is the same. For details of the anatomy of the nose please refer to *Fig.* 6.2.1. The frozen sections were thawed briefly before acetone fixation and incubation with in A) mouse MOPC21 (as an isotype control for CD19), or in B), C), D), E) and F) mouse anti-human CD19 antibody (see section 3.2.40 for full experimental details). In B) a lack of CD19$^+$ B cells is evident in the area distal to the epithelium. In C) and D) large numbers of CD19$^+$ B cells are evident in the sub-epithelium and clustering of the cells is particularly evident. In E) and F) these clusters of cells are shown at a higher magnification. Scale bars representing 50 µm are shown in A) B) C) and D) and 20 µm in E) and F).

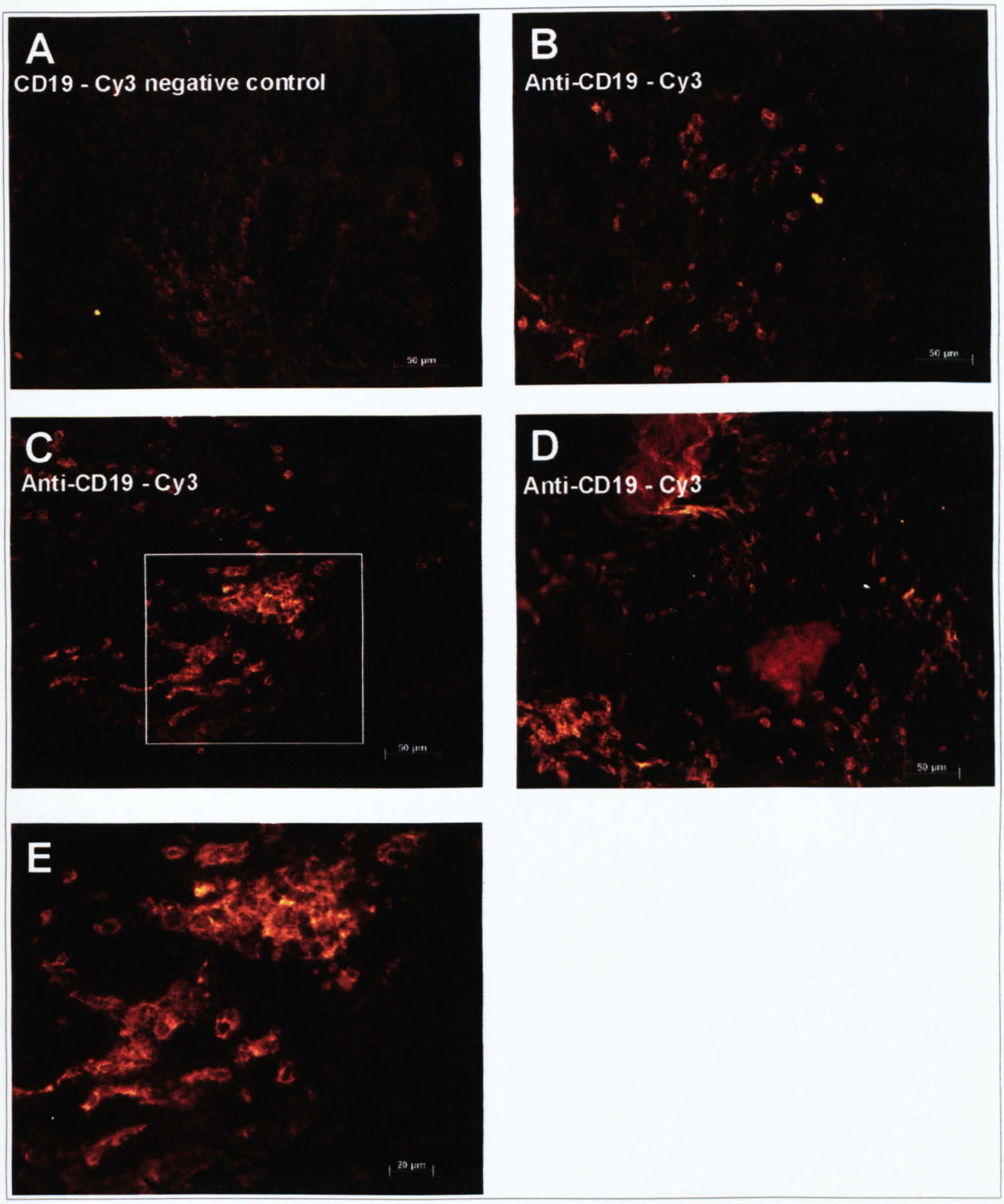


Fig. 6.4.2; Immunohistochemical fluorescent staining of CD19 using 6 µm acetone fixed frozen nasal biopsy sections. The sections presented here originated from the same biopsy as those in *Fig*. 6.4.1. The allergic status of the subject was not clear. The fluorophore Cy3 (orange) was used to immunohistochemically stain for the B cell marker CD19. In A), B), C) and E) the epithelium is to the right of the section. In D) the epithelium is not shown but the orientation of the section is the same. For details of the anatomy of the nose please refer to *Fig*. 6.2.1. The frozen sections were thawed briefly before acetone fixation and incubation with in A) mouse MOPC21 (as an isotype control for CD19 or in B), C), D) and E) mouse anti-human CD19 antibody (see section 3.2.41 for full experimental details). In B), C) and D) large numbers of CD19$^+$ B cells are evident in the sub-epithelium and clustering of the cells is particularly evident in C) and D). In E) the cluster of CD19$^+$ B cells from C) is shown at a higher magnification. Scale bars representing 50 µm are shown in A) B) C) and D) and 20 µm in E).

6.5 Analysis of V_H-Cε sequences amplified by *Pfu* from adjacent samples of the nasal mucosa from four allergic rhinitis patients.

As an alternative experimental approach, the RT-PCR amplification of V_H-Cε sequences by *Pfu* DNA polymerase from adjacent areas of the allergic nasal mucosa was undertaken. It was intended that this would enable how widely clonally related IgE$^+$ B cell clones were distributed across the tissue.

Adjacent samples of the nasal mucosa from four allergic rhinitis patients were analysed (see Table 6.5.1 for patient details). The tissue samples were of three different forms, adjacent halves of a nasal biopsy (SJ24, part A and B) and adjacent double biopsies, taken no more than 2 mm apart in the nasal mucosa, (TL25A and B and CA30A and B). In addition, a random piece of tissue excised from a whole inferior turbinate (obtained as a by-product of routine surgery) was divided in two, each resultant piece being of a similar size to a nasal biopsy (SLT1A and B).

Patient	Age	Sex	Status at sampling[a]	Total serum IgE (IU / ml) (Norm=3–150)	Specific serum IgE (IU / ml) (Norm < 0.35)[b]	Allergies[c]
SJ24	36	F	O / P	1264	HDM = 11.4 G = 58.5 C = 29.3	ND (severe eczema)
TL25	27	M	I / P	304	G = 13.1 C = 3.24	G, M, T, B, C, D
CA30	26	F	O / P	1025	HDM = 0.36 G = 101	G, D
SLT1	38	F	O / P	ND	ND	HDM, G, B

Table 6.5.1: Clinical data from patients SJ24, TL25, CA30 and SLT1. **a)** In-season (I), Out-of-season (O), Perennial (P). SL was the only patient to undergo a turbinectomy. **b)** Determined by RAST for the allergens; house dust mite (HDM), Mixed grass (G), Tree(T), Cat (C), Dog (D). **c)** Determined by a skin-prick test ≥ 2mm diameter for; *Dermatophagoides* (house dust mite) (HDM), *Phleum pratense* (grass pollen) (G), mugwort (M), three trees (T), silver birch (B), cat (C), dog (D), horse (H), *Aspergillus fumigatus* (AF), *Cladosporium herbarum* (CL), *Alternaria alternata* (A). ND represents data that could not be determined.

Twelve, fourteen or fifteen V_H-Cε sequences, all containing mutations from the germline in the V_H region were isolated from each of the eight samples of the nasal mucosa (Table 6.5.2). Sequence analysis demonstrated that in each of the four allergic rhinitis patients, none of the V_H-Cε sequences isolated from one half of the nasal mucosa sample were either duplicated or related to that isolated from the adjacent sample.

Patient	Unique sequences isolated from nasal biopsy samples	Predicted germline V_H gene usage	% Mutation from germline	Number of identical sequences isolated
SJ24 A	C1	4-30.4	3.3#	13
	C3	4-30.4	3.3#	1
	C17	4-30.4	3.6	1
SJ24 B	C1	3-30	8.1	14
	C14	4-61	ND	1
TL25 A	C17	1-02	7.4	1
	C13	3-23	5.2	3
	C11	3-53	5.6	11
TL25 B	C8	5-51	5.9	15
CA30 A	C3	5-51	11.1	2
	C3b	5-51	13.0	1
	C4b	5-51	7.5	1
	C5	5-51	5.2	1
	C8	5-51	6.2	1
	C11	5-51	10.5	2
	C12	5-51	6.3#	1
	C16	5-51	6.3#	1
	C17	5-51	10.0	1
	C20	5-51	9.6	1
	C22	5-51	10.3	3
CA30 B	C7	3-07	5.6	1
	C5	4-ND	8.4	7
	C8	4-30.1/4-31	3.3	2
	C19	4-30.1/4-31	9.1	4
SLT1 A	C4	3-11	5.6	11
	C3	4-59	7.0	3
SLT1 B	C1	1-18	14.8	12

Table 6.5.2: V_H gene usage and % V_H mutation of V_H-Cε sequences isolated from nasal biopsies from allergic rhinitis patients SJ24, TL25, CA30 and SLT1. SJ24A and B were halves of the same biopsy. Both TL25A and B and also CA30A and B were each sets of double biopsies. SLT1A and B were adjacent pieces of tissue, equivalent in size to a nasal biopsy, but taken from a whole inferior turbinate, a by-product of routine surgery. V_H gene usage is detailed such that a gene mapped to the locus 5-51 is a V_H5 gene. V_H genes whose germline sequence could not be reliably identified were denoted ND. Sequences highlighted in red represent related B clones isolated from that patient. Sequences denoted # exhibited the same degree of mutation from the germline, although the pattern of mutation differed in each clone.

A family of related IgE^+ B cells were isolated from part A of the nasal mucosa of patient SJ24 (*Fig.* 6.5.1). It was possible that related B cell clones had been present in SJ24B but that they were not effectively amplified by the PCR, for example, the abundance of V3-30 and V4-61 transcripts detected in SJ24B, may have resulted in their preferential amplification to the detriment of the amplification of other transcripts. Further investigation was therefore carried out to see if transcripts from B cells related to those identified in SJ24A could be isolated if the amplification of transcripts from SJ24B was more specifically targeted.

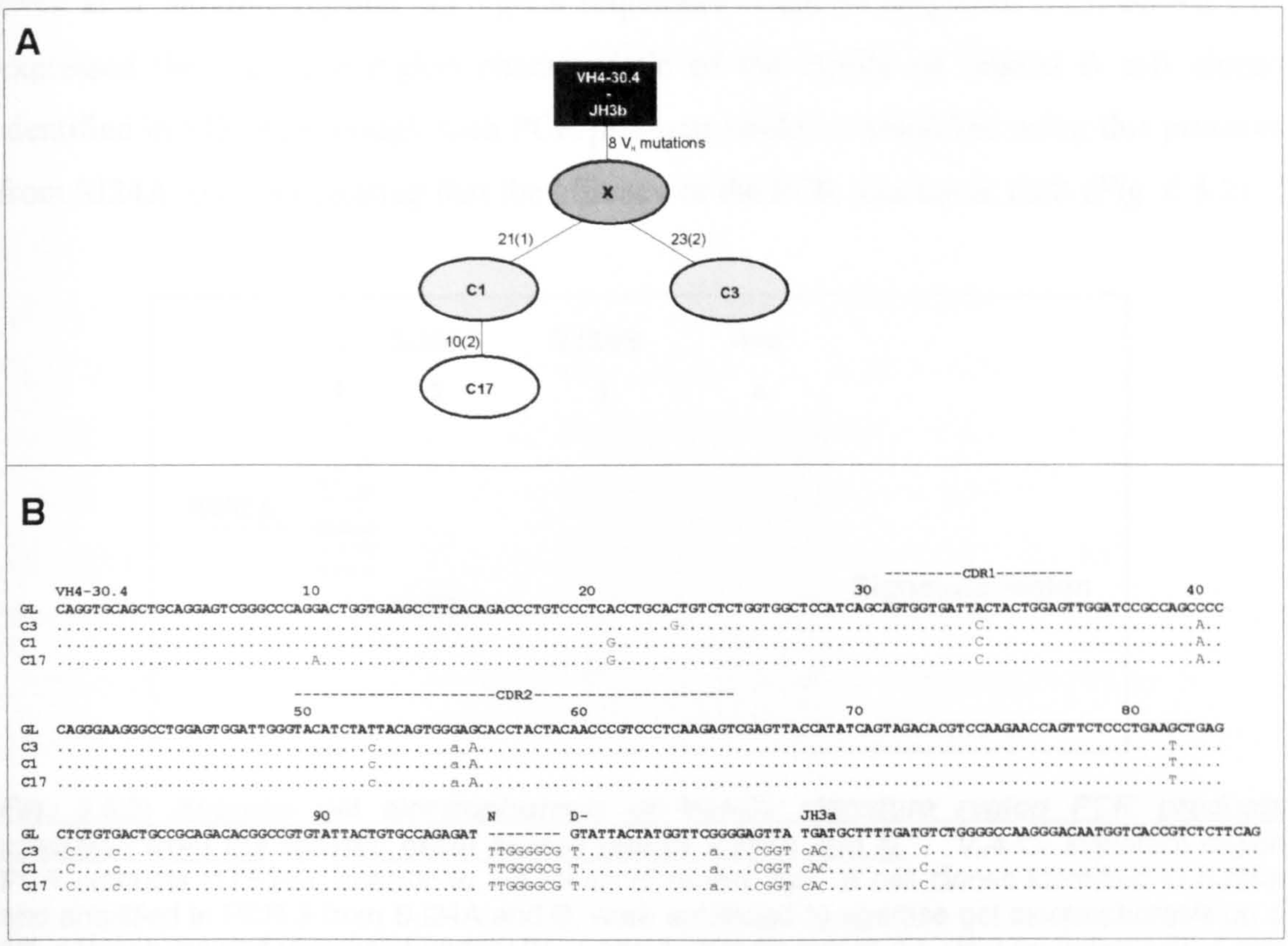


Fig. 6.5.1; Family of related IgE^+ B cell clones isolated from the nasal mucosa of patient SJ24, sample A and the V_H-D-J_H sequences. **A)** V_H4 family expressing genes mapped to the locus VH4-30.4, an unidentified D gene and JH3a. The closest germline homology as determined by VBase is depicted in a black box. Related clones are depicted as ovals. Mutations are detailed such that 10(2) represents a mutation at codon 10, position 2. **B)** The sequences of the related B cell clones are detailed. GL represents the closest germline gene homology as determined by VBase. Primer binding regions are underlined and no mutations occurring in these regions were included. Homology to the germline is represented by a dot, whereas mutations from the germline are depicted either in upper case (if causing an amino acid replacement), or lower case (if having a silent effect upon the amino acid). The mutations indicative of links between the clones are highlighted in red. The CDR regions are detailed. The N region constitutes non-templated nucleotide insertions.

Following an adapted version of the RT-PCR protocol applied in chapter 5, SJ24 B was subjected to nested PCR1 and 2 to specifically amplify V_H4-Cε transcripts (see section 3.2.20 for experimental details). These PCR products were extracted from an agarose gel and a sample carried through into PCR3. In PCR3 a primer homologous to the unique V_H-D junction was used in combination with the inner Cε specific primer to amplify the full signature region sequence from any IgE clones present in SJ24B that were related to the family of IgE$^+$ clones identified in SJ24A.

Even after multiple repeats, no V_H-Cε sequences could be amplified from SJ24B that expressed the signature region characteristic of the family of related B cell clones identified in SJ24A, although such PCR products could be amplified using this protocol from SJ24A itself, suggesting that the efficacy of the PCR was not at fault (*Fig.* 6.5.2).

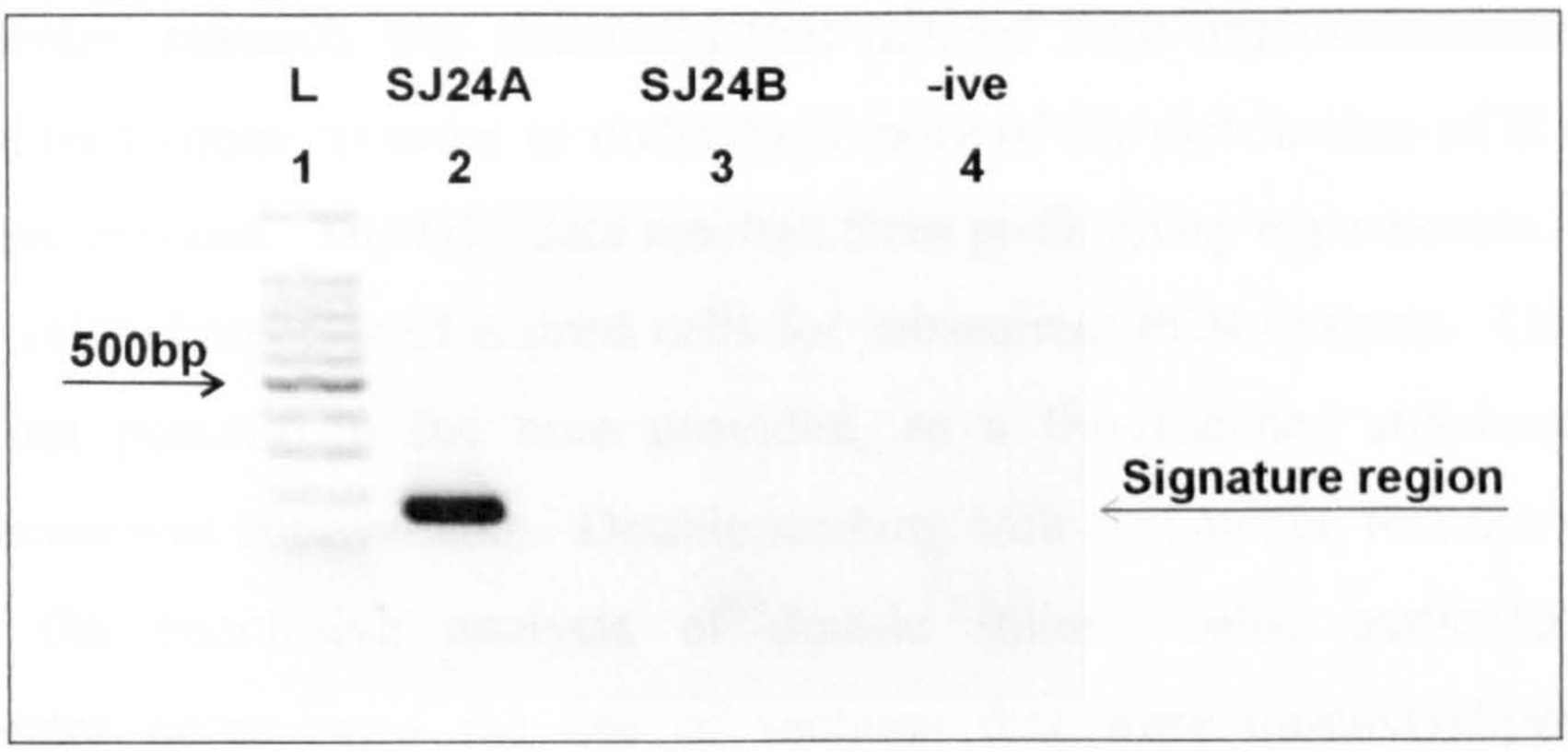


Fig. 6.5.2; Agarose gel electrophoresis of V_H4-Cε signature region PCR products amplified from the allergic nasal biopsy pieces SJ24A and B. V_H4-Cε signature region PCR products (175 bp), specific to the family of related IgE$^+$ B cell clones identified in SJ24A and amplified in PCR 3 from SJ24A and B, were subjected to agarose gel electrophoresis on a 1% agarose gel and visualised under UV. **Lane 1**; 100 bp ladder (L), (500 bp indicated). **Lane 2**; V_H4-Cε signature region PCR products amplified from SJ24A. **Lane 3**; V_H4-Cε signature region PCR products amplified from SJ24B. **Lane 4**; V_H4-Cε negative control (no cDNA).

Interestingly, in the course of this experiment, the PCR to amplify V_H-Cε sequences from SJ24A was repeated and a further fifteen sequences analysed. This illustrated that while twelve of the further fifteen sequences from SJ24A were identical to that isolated previously, two additional, unique sequences were detected (data not shown). While both were still unique to SJ24A, in future, if more detailed work is carried out, the

analysis of PCR products from repeated PCRs, may be of use in yielding sequences from a wider range of clones.

Investigation of the sequences amplified from the pair of adjacent nasal biopsies from CA30, A and B, was also undertaken in greater detail. Initial sequence analysis suggested that at least eleven distinctly different V_H5 B cells were present in one of the two adjacent biopsies (CA30A). In contrast, no V_H5 sequences were amplified from the adjacent biopsy (CA30B), (see Table 6.5.2). Interestingly, even when V_H5-Cε transcripts were specifically targeted for RT-PCR amplification from CA30B, in repeated experiments no V_H5-Cε PCR products resulted (data not shown).

6.6 Discussion.

In this chapter, research was presented that utilized both immunohistochemical and PCR based techniques, in order to understand more of the distribution of B cells in the allergic nasal mucosa. The IHC data resulted from preliminary experiments designed to enable the microdissection of stained cells for subsequent PCR analysis. Unfortunately this was not possible in the time provided, as a fluorescence attachment for the microdissector was not available. Double staining with chromogen was not sufficiently clear for the conclusive analysis of double stained cells, particularly as the microdissector necessitated the use of sections that were un-coverslipped, further impairing the quality of the image.

Microdissection of single IgE$^+$ B cells would have enabled the sequences of clones known to originate from documented areas of the nasal biopsy to be determined. However, the preliminary experiments carried out to optimise the IHC techniques for microdissection generated such interesting data that a larger, more comprehensive study should be carried out in the future. The IHC demonstrated that both CD19$^+$ and CD138$^+$ B cells were situated in the cell rich sub-epithelial layer described in *Fig.* 6.2.1 [Mygind *et al.*, 1996]. Other IgE$^+$ non-B cells, presumably mast cells, appeared to be situated both in the sub-epithelial layer and also deeper in the lamina propria. This was presumably a reflection of the more migratory nature of such cells.

The IHC confirmed an earlier documentation of IgE$^+$ plasma B cells in the allergic nasal mucosa. The sections used for these experiments were from a nasal biopsy taken outside the grass pollen season. The patient's medical details were not comprehensive enough to determine if they were grass pollen mono-allergic, but it is possible that a greater number of IgE$^+$ plasma cells (comparable with the eight IgE$^+$ plasma cells per mm^2 of the nasal mucosa from an in-season grass pollen allergic patient observed by KleinJan *et al.*, 2000) may have been detected in a biopsy taken within the grass pollen season, for while continual local IgE production is maintained in the nasal mucosa [Smurthwaite *et al.*, 2001], stimulation with allergen has been shown to increase the number of B cells undergoing ε germline transcription [Durham *et al.*, 1997].

A more comprehensive study would be needed before any sensible estimates of different B cell numbers in a nasal biopsy could be made, but it is possible that there are relatively few IgE$^+$ plasma B cells present in the allergic nasal mucosa. Durham *et al.* suggested that relatively few IgE$^+$ B cells may terminally differentiate to become IgE$^+$ plasma B cells, but that these plasma cells may secrete high levels of IgE [Durham *et al.*, 1997]. This would correlate with the finding that the quantity of IgE produced locally in the allergic nasal mucosa is sufficient to maintain the permanent sensitisation of local mast cells, without the contribution of any non-locally produced IgE [Gould *et al.*, 2003].

Furthermore, while the allergic status of the subject was unclear, preliminary IHC demonstrated that CD19$^+$ B cells occurred in clusters as well as individually within the lamina propria. It would be fascinating to expand this study to determine if this phenomenon is reproducible in further allergic and / or normal subjects and if so, whether such clusters occur only in particular regions of the inferior turbinate. In the future, double staining of the allergic nasal mucosa for CD19$^+$ IgE$^+$ B cells (intended to be encompassed within this study) should be completed. If IgE$^+$ CD19$^+$ B cells were shown to occur in clusters in the nasal mucosa, this would confirm the hypothesis suggested in chapters 4 and 5, that the sampling of clusters of B cells in the inferior turbinate by biopsy resulted in the detection of families of related B cell clones in some patients, but not others. Furthermore, it may be that while sequences from related B cell clones originated from small clusters of CD19$^+$ B cells, individual sequences may have included those from either CD19$^+$ IgE$^+$ or CD138$^+$ IgE$^+$ B cells. To date, there has been

one further report of clusters of lymphocytes occurring in the allergic nasal mucosa, but the composition of these clusters was not described and no pictorial evidence was provided to enable comparison [Fokkens *et al.*, 1990].

The identity of such clustered CD19$^+$ cells whether memory B cells, or centrocytes such as those found in germinal centres for example, could also be determined by further IHC. In addition, as such clusters of B cells might be associated with focal points of local SHM and CSR, it would be interesting to analyse what other cell types are present. While the clusters do not initially appear as organised as conventional germinal centres, the presence or absence of T cells and antigen presenting cells, even follicular dendritic cells, in or around these B cell clusters would be important to determine. It would also be of importance to determine if these cells were proliferating (by the use of the Ki67 antibody (Dako), for example).

Future work may also enable staining for AID, known to be required for both CSR and SHM [Muramatsu *et al.*, 1999], [Muramatsu *et al.*, 2000], [Revy *et al.*, 2000], [Fagarasan *et al.*, 2001] and whose protein expression would be expected at sites of local CSR and SHM (see chapter 8). This IHC could be made easier by the use of paraffin sections (enabling better tissue morphology to be preserved), as microdissection would no longer be the ultimate goal. The expansion of this work will be of particular importance, as while local germinal centres have been reported in the synovial tissue of rheumatoid arthritis patients [Randen *et al.*, 1995] and also after antigen challenge in the murine lung [Chvatchko *et al.*, 1996], no germinal centre-like structures have previously been reported in the human allergic mucosa.

In addition to the IHC, a RT-PCR approach was carried out to obtain greater knowledge of the distribution of clonally related IgE$^+$ B cells in the allergic nasal mucosa. Adjacent pieces of the allergic mucosa from four allergic rhinitis patients were examined to determine if V_H-Cε sequences suggested the presence of related B cell clones across a wide or restricted geographical area. Comparisons were made between sequences from halves of the same biopsy, adjacent nasal biopsies and also adjacent pieces of mucosa excised from a whole inferior turbinate.

When the V_H-Cε sequences amplified from these pieces of tissue were analysed, no sequences isolated from one piece of tissue were either identical or related to that in the adjacent sample. Furthermore, in patient SJ24 a B cell clonal family comprising three related B cell clones was identified in one half of the nasal biopsy (A), although even when the opposing half of the biopsy (B) was specifically probed multiple times, by a PCR to amplify the signature region unique to this B cell family, no related B cell clones could be identified in the adjacent half. Also, while sequences from eleven distinct V_H5 IgE$^+$ B cells were amplified from one nasal biopsy (CA30A), even when specifically targeted for amplification by PCR, no V_H5-Cε sequences could be identified in the adjacent biopsy (CA30B).

These results suggest that related IgE$^+$ B cells exist in an extremely localised manner, consistent with the hypothesis that clusters of B cells exist in the nasal mucosa. Furthermore, as the repertoire of B cells across the inferior turbinate appears diverse, the families of related B cell clones will not have been a consequence of a limited number of progenitor B cells existing in the nasal mucosa. A further detailed study of tissue taken systematically from a whole turbinate might strengthen these findings.

The RT-PCR based analysis of the distribution of related IgE$^+$ B cells in the nasal mucosa reported here, while providing a further example of local SHM and clonal expansion, additionally suggests that clonally related IgE$^+$ B cells are located within small geographical areas, a likely explanation of the inability to identify clonally related B cells from all biopsies. Furthermore, preliminary immunohistochemistry has demonstrated for only the second time, the presence of IgE$^+$ plasma B cells in the allergic nasal mucosa and for the first time, that small clusters of B cells, possibly indicative of confined germinal centre-like sites of concentrated local SHM and CSR activity also occur in the nasal mucosa.

Chapter 7

V_H gene usage and the distribution of somatic mutations across the V_H region of IgE^+ B cells in allergic rhinitis patients.

7.1 Introduction

Previous studies have revealed the importance of analysing somatic mutations in the V_H region and also V_H gene usage, in order to observe trends resulting from the selection of an antibody by its antigen [Betz *et al.*, 1993], [Snow *et al.*, 1997]. Evidence of such selection may manifest in the repeated selection of B cells using a particular V_H gene or class of genes, or exhibiting particular somatic mutations.

When a particular somatic mutation occurs frequently within the V region sequences of a population of cells, it can be classified either as an intrinsic or non-intrinsic hotspot of mutation. An intrinsic hotspot of mutation is considered to have arisen as a consequence of the intrinsic bias in the targeting of somatic hypermutation (SHM) within the V region. In the absence of antigenic selection, SHM is not random, but has a tendency towards transitions rather than transversions [Insel and Varade, 1994]. A transition results in the substitution of one purine residue (A or G) with the other, or one pyrimidine residue (C or T) with the other, while a transversion substitutes a purine for a pyrimidine, or *vice versa*.

Within these transitions and transversions, particular substitutions are favoured. These preferences been determined both by the study of mutations accumulated in passenger transgenes and also by those in sequences flanking the V region and then refined, to generate a set of values that reflect these nucleotide substitution preferences [Betz *et al.*, 1993b]. This compilation of data illustrated that the transition from C→T occurs most frequently and that the transition from G→A is also particularly highly favoured.

In addition to bias towards particular nucleotide substitutions, SHM also intrinsically favours mutation at particular hotspot motifs, including the nucleotide sequences R<u>G</u>YW, WR<u>C</u>Y and W<u>A</u> (where R= A or G, Y= C or T and W= A or T) [Rogozin and Kolchanov, 1992]. Intrinsic hotspots of mutation do not necessarily provide an increase in antibody affinity, but rather may have occurred coincidentally with another mutation in that B cell that does confer a beneficial effect on affinity [Berek and Milstein, 1987], [Jolly *et al.*, 1996].

In contrast, non–intrinsic hotspots of mutation are a direct consequence of selection by antigen because of the increase in the antibody's affinity and are therefore of particular

value in identifying amino acids that interact with antigen. Non-intrinsic hotspots of mutation are evident in a population of B cells that have been exposed to the same antigen, usually as replacement mutations and are distinguished by their failure to comply with the expected substitution preferences of SHM. This does not mean that non-intrinsic hotspots of mutation do not also occur at hotspot motifs, but rather that they exhibit a skewed direction of mutation so that a transversion, for example, may be much more common than expected [Jolly *et al.*, 1996].

Selection of B cells expressing antibodies of high affinity is usually dependent on the interaction of the antigen with the classical antigen binding site formed by the CDR loops of the V regions. The majority of somatic mutations that increase antigen affinity by replacing an amino acid therefore tend to occur in the CDRs, whereas silent mutations are prevalent in the FWR because of the need to maintain the structural integrity of the antibody. However, replacement mutations in the FWR may have an indirect effect upon the structure of the CDRs [Tramontano *et al.*, 1990], [Jolly *et al.*, 1996], resulting in non-intrinsic hotspots of mutation occurring occasionally in the FWRs. Alternatively some codons, particularly in the FWRs, may be conserved because of the structural importance of that amino acid.

When antigen binding does not occur across the classical antigen binding site, the distribution of replacement and silent mutation in selected B cells may be altered. B cell superantigens have affinity to the FWR of an antibody, and can be of bacterial, viral or endogenous origin, for example the bacterial *Staphylococcus aureus* protein A binds to the FWR of V_H3 antibodies [Sasso *et al.*, 1989]. In such instances, selection by a B cell superantigen may therefore result in a bias towards B cells using a particular class of V_H gene, such as V_H3 and a distorted distribution of somatic mutations, particularly across the FWRs.

In chapters 4, 5 and 6, V_H-Cε sequence data was presented from ten patients who had been subjected to a nasal biopsy and one patient who had undergone an inferior turbinectomy. This data appeared to suggest the frequent use of the V_H5 class of genes, usually a minor contributor to the antibody repertoire. In addition, of the four families of related IgE^+ B cell clones detailed in chapter 4, 5 and 6, two comprised clones

expressing V_H5, including the family of B cells from SO16 that exhibited both related IgE^+ and IgA^+ clones.

Previous researchers have observed a bias towards increased V_H5 usage in allergic patients (although this was disputed by Tilgner *et al.*, 1997, who analysed PBMC from allergic dermatitis patients). A V_H5 bias was observed in the PBMC of allergic dermatitis patients [Van der Stoep *et al.*, 1993] and allergic asthmatics [Snow *et al.*, 1997] and also in the spleen and the lung mucosa of allergic asthmatics [Snow *et al.*, 1995], [Snow *et al.*, 1999]. It was proposed that this V_H5 bias in allergic patients was a consequence of the selection of B cells by a B cell superantigen [Snow *et al.*, 1997], [Snow *et al.*, 1999].

In the following chapter the use of the different classes of V_H genes, especially of V_H5, by IgE^+ B cells has been analysed to examine whether selection by a B cell superantigen occurs in the nasal mucosa of allergic rhinitis patients. Particular attention has been given to the distribution of somatic mutations in the IgE V_H region in non-V_H5 compared to V_H5 IgE sequences and the presence of any apparent non-intrinsic hotspots of mutation.

7.2 V_H gene usage of IgE^+ B cells in the nasal mucosa and PBMC of allergic rhinitis patients.

V_H-Cε sequence data obtained from the nasal mucosa of the allergic rhinitis patients CD6, JB7, CM10, HD14, SO16, HD17, AP19, SJ24, TL25, CA30 and SLT1 (a total of 62 sequences, detailed in Tables 4.3.2 and 6.5.2) was pooled to enable analysis of the overall pattern of V_H gene usage. In addition, V_H-Cε sequence data obtained from the PBMC of patients CD6, JB7, CM10, HD14, SO16, HD17 and AP19 (a total of 50 sequences, detailed in Table 4.3.3) was pooled for comparison (*Fig.* 7.2.1). Sequences from identical or related clones were only included once in the analysis, representing a single V_H-D-J_H recombination event. This meant that sequences isolated from sister clones present in both the nasal mucosa and PBMC were completely excluded from the statistical analysis, as they could not be strictly classified into either the nasal mucosa or PBMC data set. This is unlikely to have altered the results of the analysis however, as

only six sequences were affected in this manner, of which four expressed V_H3, one expressed V_H4 and one expressed V_H5.

The expected use of the different V_H gene classes approximately reflects the number of genes in each class (see *Fig.* 7.2.1), such that the V_H3 class of genes occurs most frequently, being comprised of twenty-two members. Smaller classes of genes such as V_H5 (comprised of only two genes) occur much less frequently. As discussed in section 4.3, no V_H2 sequences were isolated from either the nasal mucosa or PBMC of any of the allergic rhinitis patients. While V_H2 is expressed at very low levels in normal PBMC (2.4%) [Brezinschek *et al.*, 2000], it is possible that the V_H2 primers were less efficient in the PCR than those amplifying the other V_H gene classes. It is not therefore possible to draw conclusions from the absence of V_H2 IgE^+ B cells in this study.

When the V_H gene usage by IgE^+ B cells in the allergic nasal mucosa was compared with the expected V_H gene usage, (based on the in-frame genomic rearrangements observed in PBMC from normal patients [Brezinschek *et al.*, 2000]) the increased use of V_H5 in the allergic nasal mucosa was highly significant (using the chi-squared test $p<0.005$), (see appendix B for details of all chi-squared calculations) with a dramatic increase from the expected 2.9% to the 29% usage observed in the allergic nasal mucosa. Furthermore the increase in V_H5 usage in the allergic nasal mucosa was statistically significant, even when compared with that observed in the allergic PBMC ($p<0.025$). There was no significant difference between normal and allergic PBMC V_H5 usage. The apparent decrease of V_H3 usage evident in *Fig.* 7.2.1 was also highly significant when compared to normal PBMC ($P<0.005$), but not when compared to the allergic PBMC.

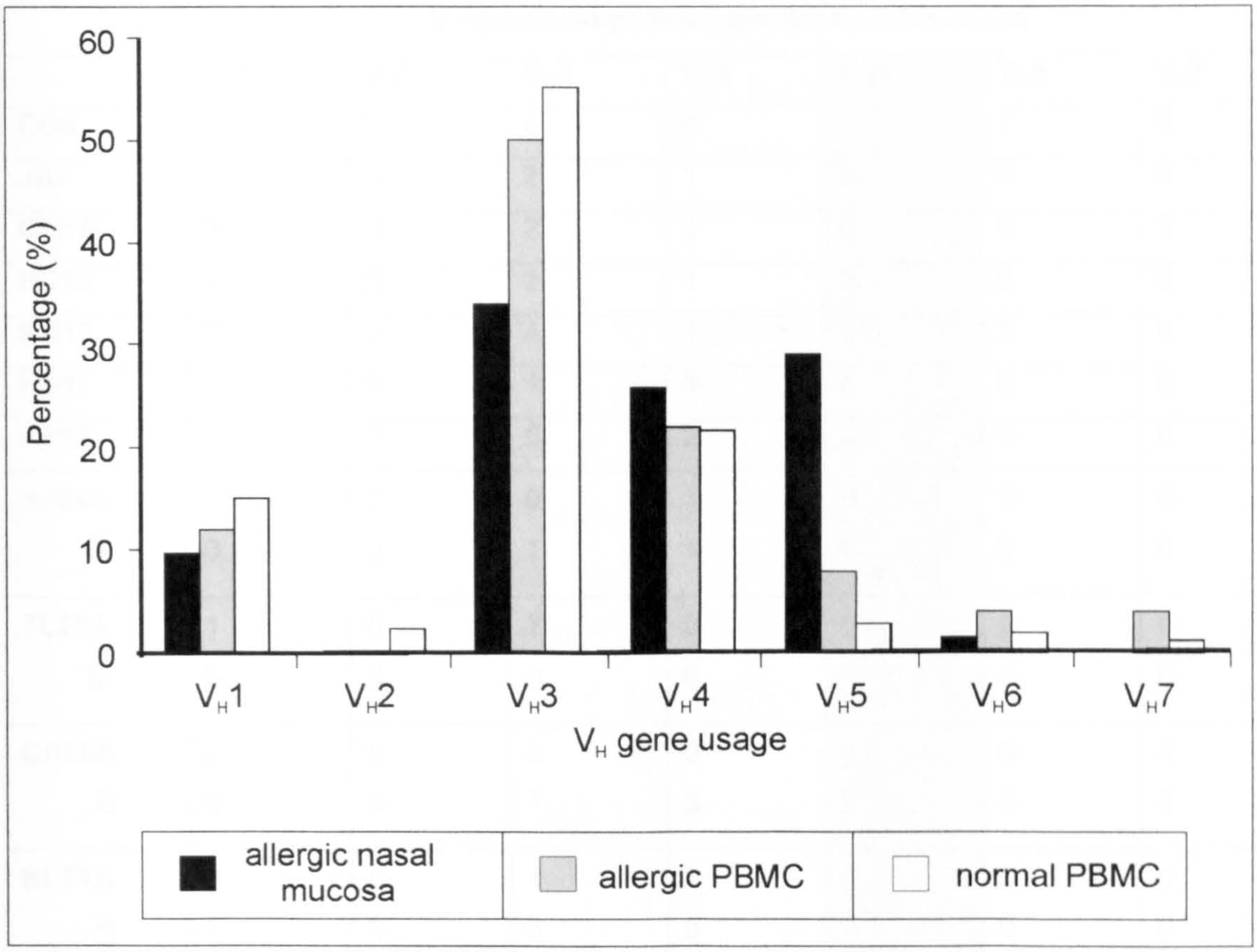


Fig. 7.2.1; V_H gene usage in IgE$^+$ B cells from allergic rhinitis patients. V_H region sequences detected by RT-PCR from IgE$^+$ B cells were analysed in order to determine the percentage usage of the different classes of V_H gene. Sixty-two sequences were pooled together from the nasal mucosa of eleven allergic rhinitis patients (black bars) and fifty sequences were pooled together from the PBMC of seven allergic rhinitis patients (grey bars). These were compared with 421 sequences previously pooled from the in-frame B cell genomic rearrangements in PBMC of normal subjects [Brezinsheck *et al.*, 2000] (white bars). Sequences from related or identical B cell clones were only included once in the analysis, representing a single V_H-D-J_H recombination event.

Although the over-representation of V_H5 in the nasal mucosa of the allergic rhinitis patients was clear in *Fig.* 7.2.1, the chart resulted from pooled data from the cohort of patients (in order to be able to analyse sufficient sequences). When the origin of the sequences was analysed, the incidence of V_H5 sequences was distributed in an even manner across PBMC samples from different individuals (although only four V_H5 sequences were detected, one in each of four out of a total of seven samples analysed). In contrast there was considerable variation in the incidence of V_H5 sequences detected in the different nasal biopsies (Table 7.2.1), as the eighteen V_H5 sequences were isolated from only five of the eleven patients.

	V_H genes amplified from the nasal mucosa						
	V_H1	V_H2	V_H3	V_H4	V_H5	V_H6	V_H7
CD6	0	0	4	0	0	1	0
JB7	1	0	2	1	0	0	0
CM10	0	0	2	0	0	0	0
HD14	1	0	3	1	**3**	0	0
SO16	0	0	4	1	**1**	0	0
HD17	1	0	1	5	0	0	0
AP19	1	0	0	2	**2**	0	0
SJ24A	0	0	0	1	0	0	0
B	0	0	1	1	0	0	0
TL25A	1	0	2	0	0	0	0
B	0	0	0	0	**1**	0	0
CA30A	0	0	0	0	**11**	0	0
B	0	0	1	3	0	0	0
SLT1A	0	0	1	1	0	0	0
B	1	0	0	0	0	0	0

Table 7.2.1; Variation in V_H gene usage detected in RT-PCR amplified sequences from the nasal mucosa of allergic rhinitis patients. V_H region sequences were amplified by RT-PCR from the cohort of eleven allergic rhinitis patients (described in detail in chapters 4 and 6). The class of V_H gene used in each sequence (according to homology with germline determined by VBase) was listed. Sequences from related or identical B cell clones were only included once in the analysis, representing a single V_H-D-J_H recombination event. In SJ24, TL25, CA30 and SLT1, where adjoining samples of the nasal mucosa were analysed (see chapter 6 for details), the sequences detected in each part, A or B, are detailed.

In CA30A in particular, of the fifteen sequences analysed, eleven were V_H5 sequences from unrelated IgE^+ B cell clones. Even more remarkable, was the observation that in the other half of this double biopsy (CA30B), no V_H5 sequences were detected (detailed in chapter 6). When chi-squared analysis was repeated on the first seven patients (CD6, JB7, CM10, HD14, SO16, HD17 and AP19), therefore excluding the major contribution of V_H5 sequences from CA30A, the difference in V_H5 gene usage between the allergic nasal mucosa and normal PBMC was still highly significant ($p < 0.005$), although the significance between V_H5 usage in the allergic nasal mucosa and allergic PBMC was lost.

An over-representation of a particular V_H gene class may be a consequence of the affinity and selection of such B cells by a B cell superantigen. As a superantigen has affinity with the FWR of an entire class of V_H genes, the affinity of the superantigen may encompass all the members of that class. There are only two functional members of the V_H5 class, V5-51 and V5-a. Of these, only 75% of the population has one or two copies of V5-a [Cook and Tomlinson, 1995]. Snow *et al.* demonstrated that while V5-51 was used preferentially in IgM, IgA and IgG, in IgE sequences V5-51 and V5-a appeared to be used equally in the PBMC of allergic asthmatic patients with copies of both genes, consistent with superantigen selection [Snow *et al.*, 1997]. In contrast, sixteen sequences (of the seventeen that were of good enough quality for detailed analysis) isolated from the nasal mucosa of the allergic rhinitis patients in this study, expressed V5-51. However, the genomic profile of the patients was not determined as only mRNA was harvested from each sample. It is therefore possible that a smaller than expected proportion of the patients relied solely on V5-51.

7.3 The distribution of somatic mutations across the V_H region of IgE^+ B cells from allergic rhinitis patients.

When mutations in the V_H-D-J_H sequences from the allergic rhinitis patients were analysed, all of the IgE sequences amplified from the allergic nasal mucosa and the majority from allergic PBMC contained mutations within the V_H region (sequences C1 and C13 isolated from the PBMC of CM10 were identical to germline, see Table 4.3.3). In addition, when the families of related B cell clones were analysed in *Fig.* 4.3.5, there was evidence of a low level of mutation in some clones across the D and J_H region after V_H-D-J_H recombination, consistent with that observed by other researchers [Lebecque and Gearhart, 1990].

Cε region sequence analysis had demonstrated that only one mutation in approximately 4720 bp (0.02%) was likely to have been introduced experimentally (see section 4.2), considerably less than the overall level of mutation observed in the V_H region sequences (between 1.8 and 22.7% in sequences from the nasal mucosa of the cohort of seven allergic rhinitis patients, detailed in section 4.3), suggesting that the majority of the mutations were a result of somatic hypermutation.

As a consequence of the data in section 7.2, suggesting a bias towards the use of V_H5, the distribution of hotspots of mutation in the V_H region was compared between V_H5 and non-V_H5 IgE sequences. If, as previous researchers had suggested, the bias towards V_H5 was a consequence of selection of the V_H5 FWR by a superantigen [Snow *et al.*, 1997], [Snow *et al.*, 1999], this may have been reflected in a departure from the conventional distribution of non-intrinsic hotspots of mutation, with increased replacement mutation in the FWRs, possibly at the expense of that in CDR1 and 2.

The comparison of V_H5 with non-V_H5 sequences was confined to sequences isolated from the nasal mucosa, partly because the significant increase in V_H5 usage was observed in the nasal mucosa, but also because insufficient V_H5 sequences were isolated from PBMC to enable meaningful analysis. V_H5 sequences from seventeen distinct B cells were of sufficient quality for analysis. These were isolated from the nasal mucosa of five different allergic rhinitis patients (HD14, SO16, AP19, TL25 and CA30A) previously identified in chapters 4 and 6. These were compared with nineteen non-V_H5 sequences (including V_H1, V_H3, V_H4 and V_H6).

In order to identify hotspots of somatic mutation within the sequences, the percentage variability, *i.e.* the percentage of sequences acquiring a mutation that changed an amino acid, was calculated and shown in *Figs.* 7.3.1 A and B. Care was taken that the analysis was not distorted, mutations common to related B cell clones were not included multiple times, but rather each different mutation from a family of clones was included once. Any sequences isolated from the nasal mucosa that were either identical or related to those from clones in the PBMC were disregarded. Mutations demonstrated in section 4.3 to be likely to have resulted from experimental error were also not included in the analysis.

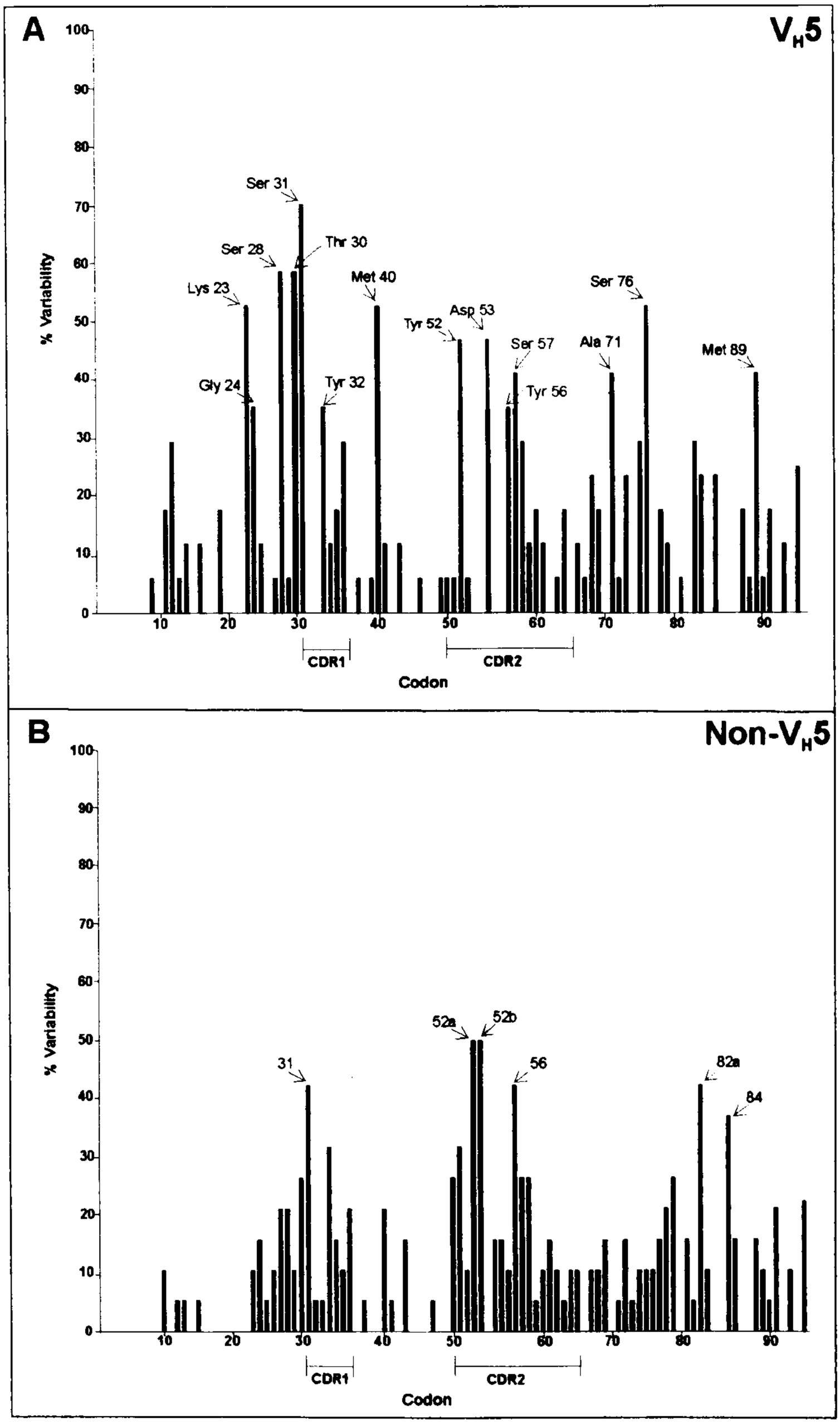


Fig. 7.3.1; Graphical representation of the distribution of replacement mutations across the V_H region sequences RT-PCR amplified from IgE$^+$ B cell clones from the nasal mucosa of allergic rhinitis patients. V_H region replacement mutations were pooled from **A)** seventeen V_H5 sequences and **B)** nineteen non-V_H5 sequences (including V_H1, V_H3, V_H4 and V_H6). The percentage variability of each codon is detailed, such that the substitution of two nucleotides, for example, within the same codon is represented as only one mutated codon. The polymorphic codons 32a, 32b, 52a, 52b, 52c, 82a, 82b and 82c are included on the codon axis and the percentage variability was adjusted to take account of the numbers of sequences that used those codons. Mutations up to and including codon 8 (the 5' primer region) were not included in the analysis. CDR1 incorporates codons 31-35 and CDR2 incorporates codons 50-65, the extents of which are both indicated on the x axis. FWR1 incorporates codons 1-30, FWR2 codons 36-49 and FWR3 codons 66-95. Apparent hotspots of mutation are detailed with the amino acid (when the same in all sequences) and the codon number.

The polymorphic codons 31a, 31b and 52b were not used in the V_H5 genes. In addition, while codon 52c is never utilised by V_H5, nor was it used by any of the non-V_H5 sequences in this data set. No mutations were detected at codons 21, 36, 45, 70 or 86 (all in the FWR) in either the V_H5 or non-V_H5 sequences, suggesting that the DNA sequence at these sites may have been cold spots, unfavourable to mutation. Alternatively, mutation of those amino acids may have been detrimental to the structural stability of the antibody (although no silent mutations, expected in this scenario, occurred either).

In the non-V_H5 sequences there was evidence of more distinct mutation of the CDRs, particularly CDR2, typical of antigen selection (*Fig.* 7.3.1 B). This was not apparent to the same extent in the V_H5 sequences in which, generally, the distinction between the CDRs and FWRs, particularly CDR2 and FWR3 was not as distinct (*Fig.* 7.3.1 A). In addition, while the V_H5 sequences exhibited a generally greater degree of variability than the non-V_H5 sequences, variability in the FWRs, particularly FWR3 of the V_H5 sequences appeared to be notably elevated in comparison, a surprising observation given its potentially destabilising effect on the structure of the antibody.

When the distribution of silent mutations across the V_H region was plotted there was no apparent difference in their distribution in the V_H5 compared to the non-V_H5 sequences (data not shown). This implied that the distribution of replacement mutations across the V_H region may be an important difference between V_H5 and non-V_H5 sequences, particularly as evidence of antigen selection would be expected to manifest mainly in the replacement mutations.

At each potential hotspot of mutation identified in *Fig.* 7.3.1, the position of the mutations in the codon was analysed to determine the precise nucleotide that was a hotspot of mutation. Hotspots of somatic mutation, evident from *Figs.*7.3.1 A and B, were then analysed in greater detail to determine if they were likely to be intrinsic or non-intrinsic in origin. The observed compared to the predicted direction of somatic mutation at each of the hotspots is detailed in Table 7.3.1. Intrinsic mutations were identified as conforming with the intrinsically favoured trends of nucleotide substitution by SHM, defined by previous data [Betz *et al.*, 1993b] and detailed in appendix C. Non-intrinsic hotspots were identified as those occurring in contrast to the expected

nucleotide substitution preferences. The intrinsic and non-intrinsic hotspots of mutation are detailed in Tables 7.3.2 (V_H5) and 7.3.3 (non-V_H5). In any instance where there were not sufficient numbers of mutations for a clear conclusion to be drawn, the hotspot was considered to be intrinsic.

Hotspot	From	To		Obs	Exp	Conclusion
V_H5						
Lys 23(1)	AAG	CAG	Gln	6	2	Biased from intrinsic trends
		GAG	Glu	2	4	
		TAG	Stop	0	2	
Gly 24(2)	GGT	GAT	Asp	0	2	Biased from intrinsic trends
		GCT	Ala	5	0	
		GTT	Val	1	4	
Ser 28(3)	AGC	AGA	Arg	3	1	Adheres to intrinsic trends
		AGG	Arg	2	2	
		AGT	Ser	4	6	
Thr 30(2)	ACC	AAC	Asn	1	1	Biased from intrinsic trends
		AGC	Ser	4	1	
		ATC	Ile	2	5	
Ser 31(2)	AGC	AAC	Asn	7	7	Adheres to intrinsic trends
		ACC	Thr	5	4	
		ATC	Ile	0	1	
Ser 31(3)	AGC	AGA	Arg	1	1	Adheres to intrinsic trends
		AGG	Arg	0	1	
		AGT	Ser	6	5	
Tyr 32(3)	TAC	TAA	Stop	0	0	Adheres to intrinsic trends
		TAG	Stop	0	1	
		TAT	Tyr	4	3	
Tyr 52(2)	TAT	TCT	Ser	1	1	Biased from intrinsic trends
		TGT	Cys	1	4	
		TTT	Phe	5	2	
Asp 53(3)	GAT	GAA	Glu	4	2	Likely to be intrinsic
		GAC	Asp	2	4	
		GAG	Glu	1	1	
Ser 57(3)	AGC	AGA	Arg	0	1	Adheres to intrinsic trends
		AGG	Arg	0	1	
		AGT	Ser	7	5	
Ala 71(2)	GCC	GAC	Asp	0	1	Adheres to intrinsic trends
		GGC	Gly	0	1	
		GTC	Val	6	4	
Ser 76(2)	AGC	AAC	Asn	6	4	Adheres to intrinsic trends
		ACC	Thr	1	2	
		ATC	Ile	0	1	
Met 89(3)	ATG	ATA	Ile	4	3	Although all substitutions would result in Ile, likely to be intrinsic
		ATC	Ile	0	2	
		ATT	Ile	1	0	
Non-V_H5						
Ser 31(2)	AGC	AAC	Asn	5	3	Adheres to intrinsic trends
		ACC	Thr	0	2	
		ATC	Ile	0	0	

Table 7.3.1; The direction of mutation at the hotspots of mutation identified in the V_H5 and non-V_H5 IgE sequences from allergic rhinitis patients determined their intrinsic or non-intrinsic origin. The replacement mutations in seventeen distinct V_H5 sequences and nineteen non-V_H5 sequences were analysed (*Fig.* 7.3.1A and B). Hotspots of mutation were defined either as intrinsic (conforming to the expected direction of mutation), or non-intrinsic (highlighted in red and biased from the expected direction of mutation). The expected direction of mutation was determined on the basis of previous studies [Betz *et al.*, 1993b]. Observed values were denoted Obs and expected, Exp.

Hotspot	Type	Reason
Lys 23(1) FWR1 AAG	Non-intrinsic	Although at WA, all mutations replaced Lys, 67% gave an A→C transversion to unexpectedly generate Gln, instead of a transition to Glu (Observed previously in V_H5 but not IgE [Snow *et al.*, 1997]).
Gly 24(2) FWR1 GGT	Non-intrinsic	Although at RGYW, all mutations replaced Gly with a G→C (83%), or a G→T (17%) transversion, to unexpectedly generate Ala or Val, instead of a transition to Asp (Observed previously in V_H5 sequences of all isotypes [Snow *et al.*, 1997]).
Ser 28(3) AGC	Intrinsic	Mutations at WRCY followed that expected by the intrinsic bias of SHM.
Thr 30(2) FWR1 ACC	Non-intrinsic	Although at WRCY, all mutations replaced Thr, the majority by a C→G transversion to unexpectedly generate Ser, instead of a transition to Ile (Observed previously in V_H5, but not IgE sequences [Snow *et al.*, 1997])
Ser 31(2) AGC	Intrinsic	Mutations at RGYW followed that expected by the intrinsic bias of SHM.
Ser 31(3) AGC	Intrinsic	Mutations at WRCY followed that expected by the intrinsic bias of SHM.
Tyr 32(3) TAC	Intrinsic	Mutations at WRCY followed that expected by the intrinsic bias of SHM.
Tyr 52(2) CDR2 TAT	Non-intrinsic	Although at WA, all mutations replaced Tyr, the majority by an A→T transversion to unexpectedly generate Phe, instead of a transition to Cys (Observed previously in V_H5 sequences of all isotypes [Snow *et al.*, 1997]).
Asp 56(3) GAT	Intrinsic	Hotspot likely to result from the intrinsic bias of SHM, but analysis of more mutations would make this more certain.
Ser 60(3) AGC	Intrinsic	Mutations at WRCY followed that expected by the intrinsic bias of SHM. (Identified previously as non-intrinsic in V_H5, but not IgE sequences [Snow *et al.*, 1997]).
Ala 71(2) GCC	Intrinsic	Mutations at WRCY followed that expected by the intrinsic bias of SHM. (Although previously identified as non-intrinsic in V_H5 IgE sequences [Snow *et al.*, 1997]).
Ser 76(2) AGC	Intrinsic	Mutations at RGYW followed that expected by the intrinsic bias of SHM.
Met 89(3) ATG	Intrinsic	Hotspot likely to be intrinsic, although not possible to determine with certainty as all possible mutations have a silent effect.

Table 7.3.2; Intrinsic and non-intrinsic hotspots of mutation identified in V_H5 IgE sequences from allergic rhinitis patients. The replacement mutations in seventeen distinct V_H5 sequences were analysed (*Fig.* 7.3.1A). Hotspots of mutation identified from this analysis were defined as either of intrinsic or non-intrinsic origin according to the criteria detailed in the text. R=A or G, Y=C or T and W=A or T in the hotspot motifs. Non-intrinsic hotspots are highlighted in red. The corresponding findings of previously identified non-intrinsic hotspots in allergic patients are detailed.

Hotspot	Type	Reason
Ser 31(2) AGC	Intrinsic	Mutations at RGYW followed that expected by the intrinsic bias of SHM.

Table 7.3.3; Intrinsic hotspot of mutation identified in non-V_H5 IgE sequences from allergic rhinitis patients. The replacement mutations in nineteen distinct non-V_H5 sequences were analysed (*Fig.* 7.3.1B). Hotspots of mutation identified from this analysis were defined as either of intrinsic or non-intrinsic origin according to the criteria detailed in the text. No non-intrinsic hotspots were identified from these sequences. R=A or G, Y=C or T and W=A or T.

The hotspots at both Met 40 and Tyr 59 in the V_H5 sequences and codon 52a, 56 and 84 in the non-V_H5 sequences (identified in *Fig.* 7.3.1), were not regarded as genuine hotspots as the mutations were evenly spread across each codon and were not significant as hotspots at the individual positions within each codon. Furthermore, the mutations had very different effects on the codons and were therefore unlikely to have been selected by antigen. In addition, the apparent hotspots of mutation at codons 52b and 82a in the non-V_H5 sequences were also dismissed. Mutations at codon 52b occurred in both of only two sequences that used the polymorphic codon and mutations at 82a were divided between Asn and Ser residues so that there were not sufficient mutations at each to determine reliably the direction of mutation.

Importantly, four potential non-intrinsic hotspots of mutation were identified by this analysis, all were evident only in the V_H5 sequences, consistent with the V_H5 sequences originating from B cells with affinity for a limited number of antigens, in comparison with the non-V_H5 sequences that would be expected to demonstrate affinity for a broader repertoire of antigens. Of these non-intrinsic hotspots, three occurred in FWR1 (Lys 23(1), Gly 24(2) and Thr 30(2)) and only one in a CDR (Tyr 52(2) in CDR2). This was in contrast to the expected effect of conventional antigen selection across the CDRs.

Interestingly, the four non-intrinsic hotspots identified in this study correlate with those previously identified in allergic asthmatic patients. Both Gly 24(2), and Tyr 52(2) were observed to be non-intrinsic hotspots in V_H5 sequences from all antibody isotypes in the PBMC of allergic asthmatic patients, whereas Lys 23(2) had only previously been identified as a hotspot in V_H5 sequences from IgA sequences in these allergic PBMC.

Thr 30(2) had also only previously been identified as a non-intrinsic hotspot in IgG and IgA V_H5 sequences from the allergic PBMC [Snow *et al.*, 1997]. However, the hotspot at Ala 71(2), identified previously from V_H5 IgE sequences as non-intrinsic [Snow *et al.*, 1997], was identified as a hotspot and displayed a similar direction of mutation in this study, but in this study was considered to be consistent with the *intrinsic* bias of SHM. In contrast, the two non-intrinsic hotspots identified from V_H5 IgE sequences isolated from allergic dermatitis patients [Van der Stoep *et al.*, 1993] at Gly 35 and Thr 95 [Betz *et al.*, 1993b] did not correlate with the findings of the present study.

Fig. 7.3.1 depicted the percentage variability of each V_H codon in the V_H5 and non-V_H5 IgE sequences. Analysis of the total percentage mutation of each codon (including silent mutations) did not demonstrate any further non-intrinsic hotspots (such as the unexpected repeat occurrence of a silent mutation, for example, data not shown). In addition, no further non-intrinsic hotspots were detected in either the V_H5 or non-V_H5 sequences when the percentage variability of each nucleotide was analysed (data not shown).

7.4 The distribution of replacement and silent mutations in the CDR and FWR of V_H region sequences isolated from IgE^+ B cells from the nasal mucosa of allergic rhinitis patients.

Analysis of R/S values (replacement / silent mutations) can provide a more detailed insight into the distribution of somatic mutations. Such analysis was therefore applied to the CDR and FWR to investigate further the apparent difference in the distribution of replacement mutations across the V_H5 and non-V_H5 V_H-Cε sequences in the nasal mucosa of allergic rhinitis patients. The same sequence data utilised in sections 7.2 and 7.3 was used for this analysis. CDR data was generated from mutations in CDR1 and 2. FWR data was generated from mutations in FWR1, 2 and 3.

In the absence of antigen selection the inherently more mutable CDRs exhibit a R/S value of 2.9, compared to the less mutable FWRs in which an R/S value of 1.5 would be expected. Conventional antigen selection results in an increase in the R/S value of the CDRs to >2.9 [Shlomchlik *et al.*, 1987], [Chang and Casali., 1994]. The non-V_H5 sequences exhibited R/S values consistent with conventional antigen selection (Table

7.4.1) displaying a significant difference between the CDR and FWR ($p < 0.025$ when the chi-squared test with Yates correction for continuity was applied). In contrast, there was no significant difference between the R/S values in the CDR and FWR of the V_H5 sequences (Table 7.4.1), implying a lack of antigen selection across the CDR and increased replacement mutations selected in the FWR.

	R/S value and (actual numbers of mutations)	
	V_H5	**Non-V_H5**
CDR	**2.38** (76 / 32)	**3.46** (83 / 24)
FWR	**2.06** (146 / 71)	**1.67** (107 / 64)

Table 7.4.1; Replacement / silent amino acid mutation (R/S) values in the CDR and FWR of V_H5 and non-V_H5 sequences from IgE^+ B cells from the nasal mucosa of allergic rhinitis patients. Seventeen V_H5 and nineteen non-V_H5 sequences were each pooled and the R/S values for the CDR and FWR of each data set calculated. A significant difference between the CDR and FWR R/S value was found in the non-V_H5 sequences ($p < 0.025$ as determined by chi-squared analysis with Yates' correction for continuity). There was no significant difference between the R/S value in the CDR compared to the FWR of the V_H5 sequences. The CDR category includes mutations from the germline as determined by the VBase database in CDR1 and 2 and the FWR category, mutations in FWR1, 2 and 3.

The R/S values detailed in Table 7.4.1 were generated by pooling all the mutations observed in the sequences belonging to each data set. Analysis of the R/S values in the individual sequences was also carried out. This demonstrated that both the V_H5 and non-V_H5 data sets were comprised of sequences with widely ranging individual R/S values, (Table 7.4.2) even in sequences from the same patient in which B cells would have been broadly exposed to the same repertoire of antigens.

When the individual sequences were categorised on the basis of their CDR and FWR R/S values, it was evident that while a greater percentage of non-V_H5 sequences showed evidence of conventional antigen selection (50.0%) (in which the CDR R/S value was >2.9 but a lower R/S value was observed in the FWR); 41.2% of V_H5 sequences also displayed such a trait. Furthermore, while 23.5% of V_H5 sequences exhibited a reversal in values, such that the FWR had a greater R/S value than the CDR, this was also observed in 16.7% of the non-V_H5 sequences.

V_H5					Non-V_H5				
Sequence		**CDR R/S**	**FWR R/S**	**Likely selective pressure**	**Sequence**		**CDR R/S**	**FWR R/S**	**Likely selective pressure**
HD14	C7	6.0	2.0	Ag	CD6	C1	3.0	2.0	Ag
SO16	C11	6.0	2.5		CD6	C13	7.0	2.5	
CA30A	C12	>6.0	2.0		HD14	C2	>5.0	2.7	
CA30A	C11	6.0	2.4		HD14	C4	4.0	1.0	
CA30A	C16	4.0	1.5		HD14	C11	3.0	1.5	
CA30A	C22	5.0	0.5		HD14	C16	5.0	1.0	
CA30A	C4b	>4.0	1.6		SO16	C13	5.0	2.0	
HD14	C12	0.3	1.7	ND	CA30B	C7	>5.0	1.7	
TL25B	C8	2.0	2.0		TL25 A	C17	3.5	1.7	
CA30A	C8	3.5	3.0		CD6	C12	1.0	2.3	ND
CA30A	C17	3.0	2.5		CD6	C2	1.5	1.4	
CA30A	C3	0.6	0.9		HD14	C9	2.3	1.2	
CA30A	C20	1.7	2.4		AP19	C16	1.0	<1.0	
HD14	C19	1.5	>6.0	SAg	CA30B	C8	>1.0	0.6	
AP19	C2	0.5	3.7		CA30B	C19	1.7	2.5	
CA30A	C5	>4.0	5.0		CD6	C5	3.0	5.0	SAg
CA30A	C3b	1.3	5.0		AP19	C3	>2.0	4.0	
					AP19	C6	1.5	3.5	

Table 7.4.2; Replacement / silent (R/S) values in the CDR and FWR of V_H5 and non-V_H5 sequences from IgE^+ B cells from the nasal mucosa of allergic rhinitis patients. The CDR included mutations in CDR1 and 2. The FWR included mutations in FWR1, 2 and 3. Sequences were categorised according to the type of antigenic selection most likely to have occurred where conventional antigen selection (Ag) induced a CDR R/S >2.9 and a difference between the CDR and FWR R/S values of at least 1.0. When antigen selection could not be determined (ND), either the sequence did not exhibit a CDR or FWR R/S value >2.9 or the difference between the values was less than 1.0. Superantigen selection (SAg) was proposed when the FWR >2.9, greater than that in the CDR and there was a difference between the CDR and FWR R/S values of at least 1.0. Where a R/S value is preceded by >, there were no silent mutations and where preceded by <, no replacement mutations.

There was no correlation in general between sequences that demonstrated R/S values suggesting superantigen selection and those that exhibited the non-intrinsic mutations identified in section 7.3. Most sequences exhibited one or two of the non-intrinsic hotspots, two exhibited three of these hotspots, while one sequence (CA30A C20) had

accumulated all four of the mutations thought to be non-intrinsic. The R/S values in CA30A C20 did not clearly suggest superantigen selection, although the R/S values were higher in the FWR than the CDR (2.4 and 1.7 respectively). The family of V_H5 IgE^+ B cell clones identified from the nasal mucosa of patient SO16 (represented by SO16 C11) did not contain any of the non-intrinsic mutations, consistent with conventional antigen selection. However, the family of V_H5 IgE^+ B cell clones identified in the nasal mucosa of AP19 (represented by AP19 C2), exhibited two of the non-intrinsic mutations, in addition to R/S values consistent with superantigen selection.

7.5 Analysis of replacement and silent mutations in the individual FWRs of V_H5 sequences from the nasal mucosa of allergic rhinitis patients.

In section 7.4, unusual R/S values in the CDR and FWR of the V_H5 sequences were identified. The apparent decrease in the R/S value of the CDRs and increase in the FWRs was consistent with superantigen selection, although no conclusive parallels could be drawn between the presence of non-intrinsic hotspots and sequences exhibiting particularly distorted overall R/S values. As a superantigen need not have contact with all the FWRs, the R/S values in the individual FWRs 1, 2 and 3 of the V_H5 sequences were therefore examined to determine if closer analysis indicated any correlation (see appendix D for data).

The combined R/S value in FWR1 was 2.16, slightly higher than overall V_H5 FWR R/S of 2.06. The combined R/S in FWR2 was however only 1.06, exhibiting the low variability expected of a FWR. In contrast, the combined R/S of FWR3 was 2.61. This suggested that the skewed R/S values observed in the overall FWR of the V_H5 sequences were likely to have resulted from the unusually high variability of FWR1 and FWR3. The particularly high R/S value of the V_H5 FWR3 did not seem to be a consequence of FWR3 being generally more mutable, as the R/S value of FWR3 in the non-V_H5 sequences was consistent with that expected (1.57). All of the sequences classified in Table 7.4.2 as subject to superantigen selection exhibited more replacement than silent mutations in FWR3, although this was also observed in some of the antibodies thought to have been selected by conventional antigen, (data not shown).

Although elevated FWR1 R/S values were also not solely confined to sequences selected by superantigen, interestingly the sequence with the most skewed overall R/S values, CA30A C3b, also exhibited the highest individual R/S value in FWR1 (R/S = 7.0), but not in FWR3. Those sequences with the highest individual FWR1 R/S values also included two of the other three sequences classified as superantigen selected (AP19 C2 and CA30A C5). In addition, CA30A C20 exhibited all four of the non-intrinsic mutations and a high FWR1 R/S value of 5.0, but a low FWR3 R/S value. (Details of the numbers of replacement and silent mutations used in calculating all R/S values are detailed in appendix D).

7.6 Discussion

In this chapter a detailed analysis of the usage of the different V_H gene classes and the distribution of somatic mutations across the V_H gene in allergic rhinitis patients was presented. Previous researchers had reported a bias towards the increased use of the V_H5 gene class in IgE^+ B cells in the PBMC of allergic dermatitis patients [Van der Stoep *et al.*, 1993] and in both the PBMC, spleen and lung mucosa of allergic asthmatic patients [Snow *et al.*, 1997], [Snow *et al.*, 1995], [Snow *et al.*, 1999]. In addition, an unusual distribution of replacement and silent mutations across the CDR and FWR of the V_H5-Cε V_H5 sequences was observed in many of these studies [Van der Stoep *et al.*, 1993], [Snow *et al.*, 1995], [Snow *et al.*, 1999]. These observations led to the suggestion that antigen selection by a B cell superantigen with affinity for the FWR of V_H5 may have occurred in these allergic patients [Snow *et al.*, 1995], [Snow *et al.*, 1999]. It was therefore of interest to determine whether such trends were observed in V_H-Cε sequences RT-PCR amplified from the nasal mucosa and PBMC of allergic rhinitis patients.

Superantigens have long been known to activate T cells by simultaneously binding to both the T cell receptor and also to MHC class II on antigen presenting cells outside of the conventional peptide-binding groove [reviewed by Proft and Fraser, 2003]. More recently however, the interaction of superantigens with B cells *via* the conserved V_H region FWRs has been observed, resulting in biased usage of the V_H gene classes. Such B cell superantigens are of similar origins to T cell superantigens, from bacterial, viral and endogenous sources. They include protein A from *Staphylococcus aureus* (SpA,

specific to V_H3) [Sasso *et al.*, 1989], protein L from *Peptostreptococcus magnus* (specific to kappa light chains) [Nilson *et al.*, 1992], HIV gp120 (specific to V_H3) [Berberian *et al.*, 1993], the human protein Fv (specific to V_H3 and V_H6) [Silverman *et al.*, 1995].

In chapters 4, 5 and 6 of this thesis, V_H5 B cell clones featured prominently. Two of the four families of related IgE^+ B cell clones (identified in chapters 4 and 6) utilised V_H5, including the family of clones that additionally incorporated IgA^+ B cell clones. The detailed analysis of V_H-Cε sequences from the allergic rhinitis patients indicated that there was a highly significant difference between the V_H5 gene usage in the allergic nasal mucosa and both that expected on the basis of the genomic rearrangement of functional V_H regions identified in normal PBMC [Brezinschek *et al.*, 2000] and also that observed in allergic PBMC. 29% of sequences isolated from the nasal mucosa used V_H5, comparable with that observed in the allergic lung mucosa (33%) [Snow *et al.*, 1999]. The use of V_H3 in the allergic nasal mucosa was highly significantly decreased compared to that in normal PBMC, possibly as a consequence of the over-use of V_H5, an observation also noted in the allergic lung [Snow *et al.*, 1999].

While other researchers identified an increased usage of V_H5 in the PBMC of allergic patients [Van der Stoep *et al.*, 1993], [Snow *et al.*, 1996], there was no significant difference in the use of V_H5 in PBMC from allergic rhinitis patients analysed in this study, suggesting that in these patients increased V_H5 use was restricted to the local microenvironment. It is possible that the V_H5 bias was locally generated in the allergic lung mucosa also, but that the greater volume of tissue meant that cells that had migrated from the lung constituted a much larger percentage of those present in the circulation.

Intrinsic hotspots of somatic hypermutation were identified in the V_H5 and non-V_H5 sequences from the nasal mucosa of the allergic rhinitis patients, reflecting the intrinsic bias of the SHM mechanism. Non-intrinsic mutations, characteristic of the selective pressures of antigen, were only identified in the V_H5 sequences, consistent with these B cell clones having affinity with a restricted repertoire of antigens. In contrast to that expected as a consequence of conventional antigen selection across the CDR loops, three of the four non-intrinsic hotspots occurred in FWR1 (Lys 23(1), Gly 24(2) and Thr

30(2)), while only one occurred in a CDR (Tyr 52(2) in CDR2). Although mutations in the FWRs occasionally have an indirect effect on the conformation of the CDRs and therefore on conventional interaction with antigen, it seems unlikely that all three hotspots would have such an effect. Interestingly, these non-intrinsic hotspots exactly correlated with those previously identified by analysis of V_H5 sequences from the PBMC of allergic asthmatics [Snow *et a*l., 1997], although not with those observed in the V_H5 sequences from the PBMC of allergic dermatitis patients [Van der Stoep *et al.*, 1993], [Betz *et al.*, 1993b].

The observation of common non-intrinsic hotspots suggests that V_H5 sequences may be selected in both allergic asthmatics and the nasal mucosa of allergic rhinitis patients by the same superantigen. A different superantigen may be responsible for the similar phenomena in allergic dermatitis patients. This may be a result of the distinction between the presence of an airborne superantigen, versus a superantigen that originates from bacteria that colonise the broken skin of dermatitis patients, for example.

In order to determine if the general distribution of replacement and silent mutations across the V_H region of the V_H5 sequences differed from that expected from conventional antigen selection, the R/S values in the CDR and FWR of the V_H5 and non-V_H5 sequences were compared. The R/S values in the non-V_H5 sequences reflected conventional antigen selection, with a significantly greater R/S value in the CDR than the FWR [Shlomchik *et al.*, 1987], [Chang and Casali, 1994]. In stark contrast, there was no significant difference between the R/S values observed in the CDR compared to the FWR of the V_H5 sequences. The interpretation of the data was complicated by the observation that individual sequences from both the V_H5 and non-V_H5 data sets exhibited a range of different R/S values, some consistent with conventional antigen selection, some in which no evidence of any selection was obvious, and some with evidence of selection across the FWRs, consistent with that of a superantigen. This was also observed when previous researchers analysed allergic PBMC [Snow *et al.*, 1997], [Van der Stoep *et al.*, 1993].

Van der Stoep suggested that such R/S values may result from chronic antigen stimulation, leading to the accumulation of mutations with a 'neutral' effect on the antibody affinity. This might skew R/S values from that expected, towards a more

random pattern [Van der Stoep *et al.*, 1993]. This would correlate with the greater degree of mutation observed in V_H5 sequences from the nasal mucosa, but would not lessen the possibility that superantigen selection had occurred. It is perhaps more likely, however, that while a superantigen initially activates V_H5 B cells, the activated B cells are allergen specific and therefore later acquire specific mutations. Alternatively, the superantigen may allow the simultaneous conventional binding of an antigen to the CDRs, as suggested in *Staphylococcus aureus* protein A (SpA) binding to V_H [Graille *et al.*, 2000]. In either such scenario, a blurred imprint of either superantigen or conventional antigen selection would result, consistent with that observed in some of the V_H5 sequences.

When the R/S values of the V_H5 sequences in FWR1, 2 and 3 were analysed separately, the distortion of the overall FWR R/S value was shown to originate from particularly high levels of variability in FWR1 and FWR3. The highest FWR1 R/S values occurred in sequences in which the overall R/S values were most skewed, particularly CA30A C3b. CA30A C20 exhibited all four of the proposed non-intrinsic hotspots and also one of the highest FWR1 R/S values.

When the sequences representing families of related V_H5 B cells were studied, the family of V_H5 B cells isolated from the nasal mucosa of patient SO16 demonstrated evidence of conventional antigen selection (as suggested to be likely from the pattern of mutation observed in chapter 5). In contrast, the family of related V_H5 B cells isolated from the nasal mucosa of patient AP19 demonstrated R/S values (and two non-intrinsic hotspots) that implied the family may have expanded in response to a superantigen.

The research presented in this chapter demonstrates that the bias towards increased usage of V_H5 sequences in allergic patients, previously identified in dermatits and asthma patients [Van der Stoep *et al.*, 1993], [Snow *et al.*, 1995], [Snow *et al.* 1999], is also present in the nasal mucosa of allergic rhinitis patients. In contrast to previous research however, the bias appears to be confined to the local nasal mucosa and is not significant in the PBMC. In addition, analysis of adjacent pieces of nasal mucosa (chapter 6) suggested that the V_H5 clones appeared to be extremely locally contained, possibly as clusters of B cells, perhaps explaining why while no V_H5 sequences were amplified from some nasal biopsies, V_H5 was predominantly amplified from others.

Taken in combination, the V_H5 bias in the nasal mucosa, the unconventional hotspots of non-intrinsic mutation and the distorted R/S values in the CDR and FWR do suggest that selection of V_H5 by the interaction of a superantigen with the FWR is likely to have occurred in the nasal mucosa of particular allergic rhinitis patients. Furthermore, it is possible that while such an interaction might predominantly be with FWR1, some contact with CDR2 and FWR3 may also occur. This model of interaction would correlate with that observed previously by B cell superantigens and V_H. SpA, for example, is known to interact with FWR1, FWR3 and CDR2 [Randen *et al.*, 1993], and FWR3 has previously been suggested to act as an alternative antigen binding site [Kirkham *et al.*, 1992].

Identifying a superantigen with affinity for the FWR of V_H5 in the nasal mucosa of allergic rhinitis patients would make a major contribution towards understanding the aggravated symptoms of allergy observed in some individuals. The superantigen may be bacterial in origin, for example IgE antibodies directed against *Staphylococcus aureus* enterotoxins (SAE) in nasal polyps were linked with an increased severity of asthma [Bachert *et al.*, 2003]. (Preliminary investigation of SAE IgE^+ nasal polyp samples did not suggest a V_H5 bias (data not shown), although the allergic rhinitis patients detailed in this chapter were not screened for such infections).

It is also possible that a superantigen could be of viral, endogenous or allergenic origin. Of all of the patients in this study, CA30 was most likely to have had exposure to a superantigen; sequence CA30A C20 contained all four of the proposed non-intrinsic V_H5 hotspots of mutation, sequence CA30A C3b demonstrated R/S values that were the most highly skewed towards superantigen selection and eleven distinct V_H5 sequences were amplified from the nasal mucosa of patients CA30. Patient CA30 was allergic to both grass and house dust mite, implying that either could be a candidate allergen to act as a superantigen.

HDM was previously suggested as a potential superantigen [Snow *et al.*, 1995], although there was no general correlation between the amplification of V_H5 sequences from the nasal mucosa and sensitivity to HDM, in the present study. However, all of the patients from whom V_H5 sequences were isolated, were allergic to grass pollen.

Four of the five patients from which V_H5 sequences were amplified were biopsied within the grass pollen season, whereas four of the six patients from whom no V_H5 sequences were amplified from the nasal mucosa (all both grass pollen and perennial sufferers) were biopsied outside the grass pollen season. In previous research in which a V_H5 bias was demonstrated in well-characterised allergic patients, the subjects have exhibited multiple allergies including grass pollen [Snow *et al.*, 1997], [Snow *et al.*, 1999]. It is therefore possible that grass pollen acts as a superantigen and that although patient CA30 was biopsied outside the pollen season, the V_H5 sequences originated from B cells stimulated earlier in the year.

It is also possible that multiple allergens could act as different superantigens in different allergic disorders. For example, a V_H1 bias and lack of conventional antigen selection has been observed in peanut allergic patients [Janezic *et al.*, 1998] and in allergic dermatitis the non-intrinsic hotspots in the over-represented V_H5 sequences do not correlate with those observed in allergic asthma and rhinitis.

Superantigen binding to the V_H5 FWR of the B cell receptor would enable non-specific activation and subsequent clonal expansion of allergen specific B cells. In addition, cross-linking of IgE on the surface of mast cells would non-specifically instigate cell degranulation, releasing mediators such as IL-4 with a consequent increase in allergen specific IgE and exacerbation of the allergic response. For example, the superantigen protein Fv is endogenous to normal liver but is released in the gut as a consequence of viral hepatitis. Some symptoms of viral hepatitis have been suggested to result from the degranulation of basophils and mast cells by the non-specific binding of protein Fv to IgE in just such a manner [Patella *et al.*, 1993]. The non-specific binding of Protein L to IgE light chains has also been shown to stimulate the secretion of IL-4 and IL-13 from basophils [Genovese *et al.*, 2003]

It is likely that similarly, in the allergic nasal mucosa the superantigen binds to the FWR of V_H5 IgE on the surface of mast cells, stimulating their degranulation, prolonging and exacerbating allergic symptoms by the positive feedback of IL-4, stimulating T_H2 cells and further switching of B cells to express IgE. The bias towards V_H5 is likely to result from the non-specific activation of B cells expressing a V_H5 antibody on the B cell surface.

The work presented in this chapter has provided many more questions to be addressed. While normal, non-allergic nasal biopsies do not contain sufficient IgE for analysis, RT-PCR amplification of V_H-Cε sequences from the PBMC or the whole inferior turbinate of non-allergic patients may be possible from carefully selected subjects and would provide more accurate 'expected' values of V_H gene usage than those from the productive genomic rearrangement used for comparison in this study (determined by [Brezinschek *et al.*, 2000]). A further study of V_H5 sequences from normal subjects may also enable the non-intrinsic hotspots identified in this study to be clearly confirmed and distinguished from mutations intrinsic to the V_H5 sequence itself.

It would then be of interest to compare the V_H gene usage in other antibody isotypes from the nasal mucosa of both normal and allergic rhinitis patients. A bias towards V_H5 should be evident in all isotypes as a superantigen has affinity for the FWR. Previous analysis of IgM in allergic lung was however unable to demonstrate a V_H5 bias [Snow *et al.*, 1999] and some of the non-intrinsic hotspots previously identified in the PBMC of allergic asthmatics, were only evident in V_H5 antibodies of particular isotypes [Snow *et al.*, 1997]. It is possible that IgM B cells activated by superantigen switch promptly to another antibody isotype, so that V_H usage by IgG or IgA antibodies in the allergic nasal mucosa might be more revealing. It would also be of interest to extend the analysis of V_H gene usage throughout the inferior turbinate, as it is possible that if the whole of the inferior turbinate were analysed a bias towards V_H5 usage would be evident in all or a particular sub-set of allergic rhinitis patients, a feature that may be disguised by the varying cellular contents of individual biopsies.

The area of particular interest for future work however would be to explore further the identity of the proposed superantigen and its manner of interaction with V_H5. This could be determined by the construction of a combinatorial Fab library of V_H5 antibodies, ideally amplified from the nasal mucosa of an allergic rhinitis patient, such as CA30, from whom evidence of local superantigen selection of B cell clones is strongest. This would enable not only the affinity of V_H5 antibodies for a variety of potential superantigens to be determined, but also the eventual crystallisation of high affinity complexes, so that the interaction of the superantigen with V_H5 and in particular with the proposed non-intrinsic hotspots, could be identified.

In conclusion, while the overall antibody response in the allergic nasal mucosa would appear to be the consequence of a multi-clonal response to multiple epitopes and antigens, the research presented in this chapter suggests that the predominance of families of related V_H5 B cells, the biased usage of V_H5, increased replacement mutations in the FWR of V_H5 sequences and unusual non-intrinsic mutations in V_H5, support the hypothesis of superantigen selection of B cells expressing V_H5 antibodies in the nasal mucosa of allergic rhinitis patients.

Chapter 8

Local expression of mRNA transcripts encoding activation-induced cytidine deaminase in the nasal mucosa of allergic rhinitis patients.

8.1 Introduction.

Activation-induced cytidine deaminase (AID) expression by active $CD19^+$ B cells has been shown to be essential in enabling class switch recombination and somatic hypermutation to take place in both murine [Muramatsu *et al.*, 1999], [Muramatsu *et al.*, 2000], [Fagarasan *et al.*, 2001] and human [Revy *et al.*, 2000] systems.

In humans, hyper IgM syndrome has been shown to result in some cases from the deficiency of functional AID protein (HIGM2). Investigation of AID expression in different human tissues suggested that AID mRNA was expressed in the human lymph nodes, tonsil and spleen. In addition however, AID expression was detected at a much lower level in most of the other human tissues tested from a panel of commercial cDNA samples, including the kidney, pancreas, and very weakly in the lung [Muto *et al.*, 2000]. As investigation of the murine gut mucosa revealed that local expression of AID corresponded with local CSR to IgA [Fagarasan *et al.*, 2001], it seemed important to determine if AID mRNA was present in the nasal mucosa of allergic rhinitis patients. Local AID expression in the nasal mucosa would be assumed to be necessary in order to support local CSR and SHM.

Time-course studies of AID mRNA expression stimulated in normal PBMC by *in vitro* incubation with anti-CD40 and IL-4 (stimuli for AID expression and CSR) were carried out in order to determine the potential value of AID mRNA as a marker for local CSR and SHM. Further RT-PCR and Southern blot analysis investigated whether AID mRNA transcripts were expressed in the nasal mucosa of allergic rhinitis patients.

8.2 Time-course analysis of AID expression in stimulated PBMC.

Previous research demonstrated that expression of AID mRNA transcripts could be induced in normal PBMC by *in vitro* incubation with anti-CD40 and IL-4 (mimicking B cell interaction with and stimulation, by T cells) [Revy *et al.*, 2000]. In these normal subjects, AID mRNA expression (undetectable at day zero) was evident after five days of incubation. Revy *et al.* also determined that AID expression remained detectable after eight or twelve days incubation, although the strength of this expression was not commented upon.

The work by Revy *et al.* was repeated and extended in this study to determine the exact time taken for AID transcripts to be generated from normal PBMC and the length of time for which the transcripts remained when constantly stimulated by IL-4 and anti-CD40 (as would also occur in the allergic nasal mucosa as a consequence of chronic antigen exposure). RT-PCR analysis of the stimulated PBMC demonstrated weak AID expression after *in vitro* incubation for just one day, although an up-regulation of AID mRNA occurred after five days in some cultures (data not shown) and considerable up-regulation was always evident at six and seven days (*Fig.* 8.2.1). This correlated with other work conducted by our group, suggesting that under the same conditions, evidence of CSR was detectable by switch circle transcript (SCT) analysis after six or seven days incubation (unpublished observations by P. Takhar).

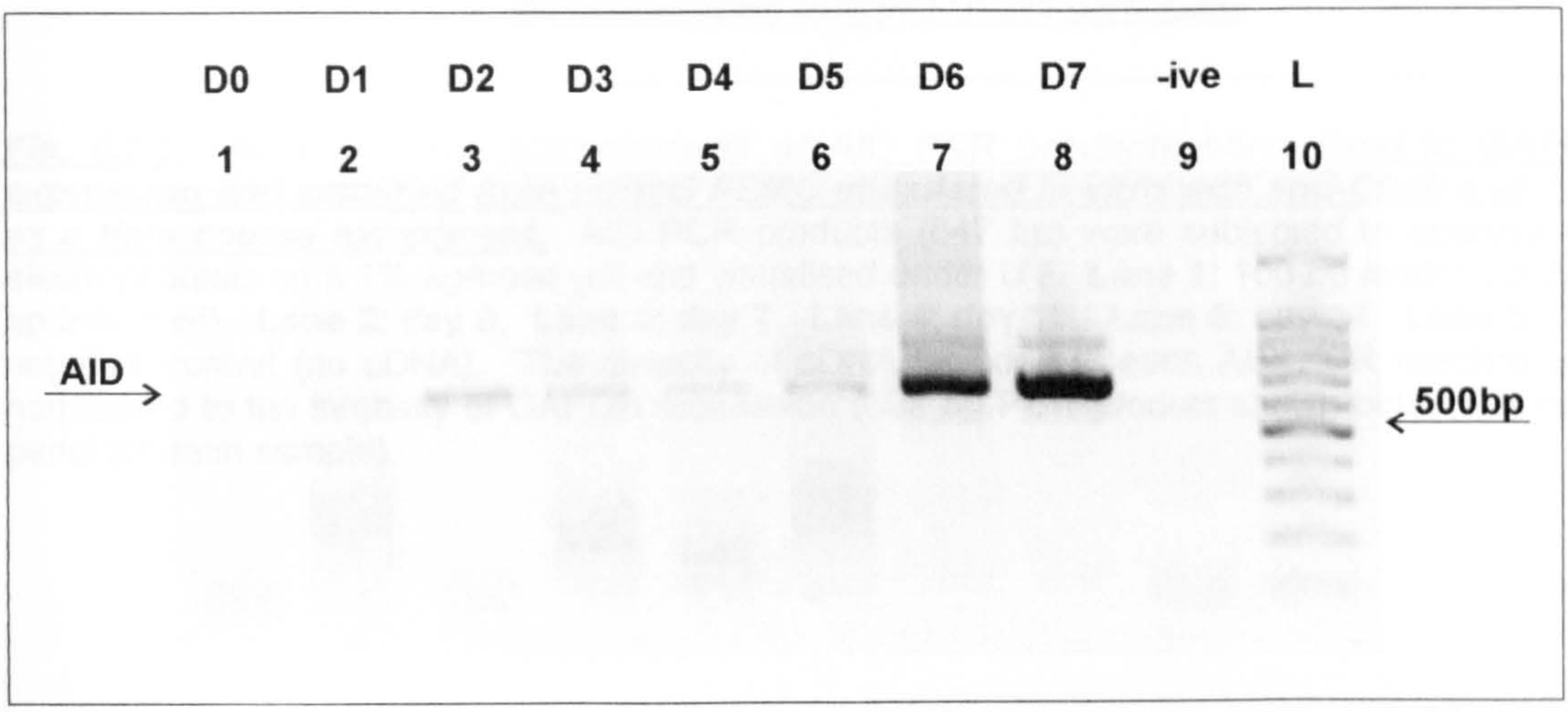


Fig. 8.2.1; Agarose gel electrophoresis of AID PCR products amplified from normal PBMC stimulated in vitro with anti-CD40 and IL-4 as a time-course experiment. AID PCR products (647 bp) were subjected to agarose gel electrophoresis on a 1% agarose gel and visualised under UV. **Lane 1**; day 0. **Lane 2**; day 1. **Lane 3**; day 2. **Lane 4**; day 3. **Lane 5**; day 4. **Lane 6**; day 5. **Lane 7**; day 6. **Lane 8**; day 7. **Lane 9**; AID negative control (no cDNA). **Lane 10**; 100 bp ladder (L), (500 bp indicated).

Additional time-course experiments demonstrated that when the cDNA from each of the stimulated PBMC samples was normalised to the expression of the GAPDH housekeeping gene (and proportional amounts of cDNA included in the PCR), AID mRNA expression was reduced, but still evident after ten days incubation (*Fig.* 8.2.2). After fourteen days, no AID expression was evident when the samples were normalised to GAPDH, although when 5 μl of undiluted cDNA was PCR amplified a low level of

expression was evident, indicating that AID expression, while considerable down-regulated, had not completely ceased (data not shown).

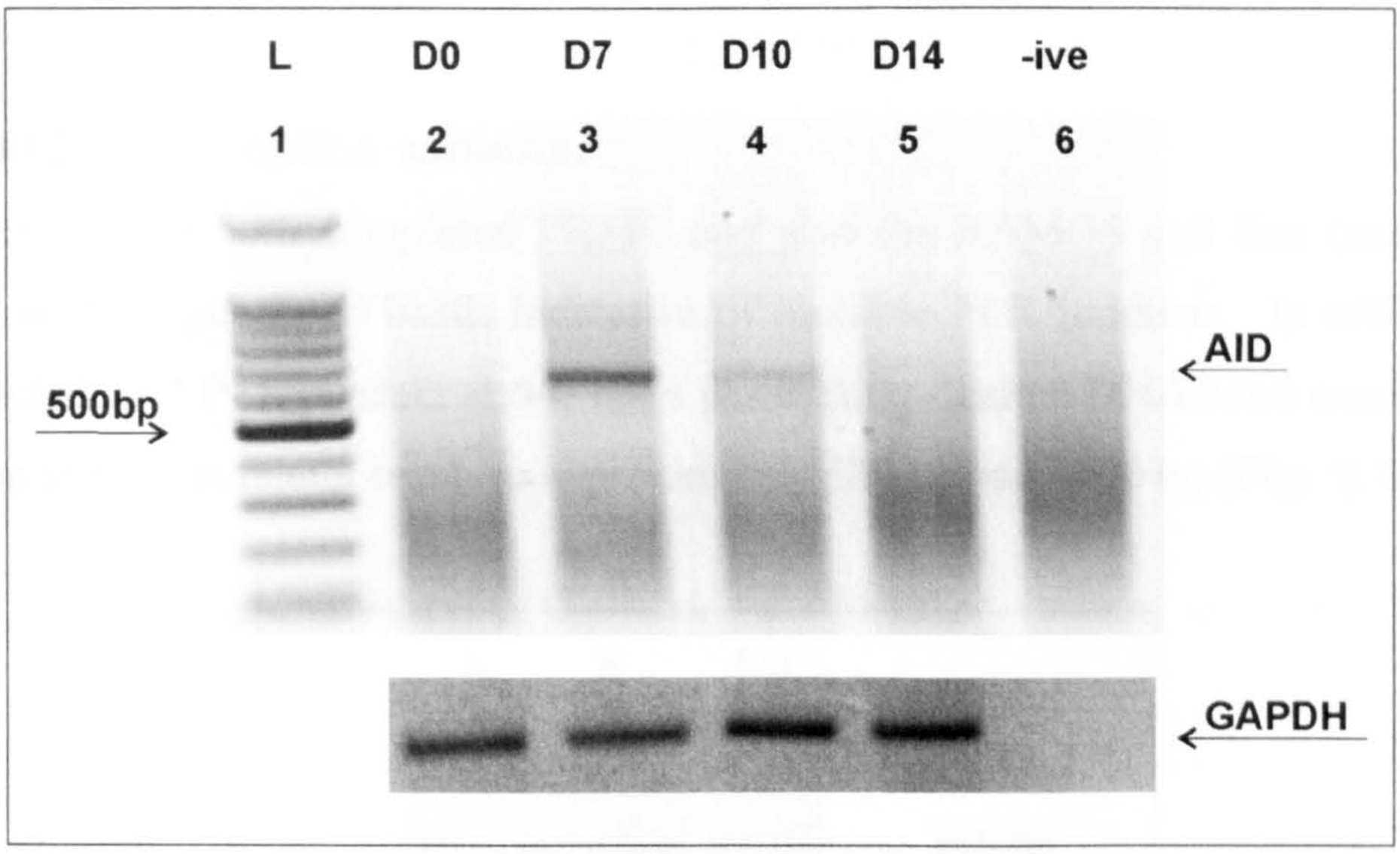


Fig. 8.2.2; Agarose gel electrophoresis of AID PCR products normalised to GAPDH expression and amplified from normal PBMC stimulated in vitro with anti-CD40 and IL-4, as a time course experiment. AID PCR products (647 bp) were subjected to agarose gel electrophoresis on a 1% agarose gel and visualised under UV. **Lane 1**; 100 bp ladder (L) (500 bp indicated). **Lane 2**; day 0. **Lane 3**; day 7. **Lane 4**; day 10. **Lane 5**; day 14. **Lane 6**; AID negative control (no cDNA). The quantity of cDNA included in each AID PCR reaction was normalised to the intensity of GAPDH expression (393 bp PCR product shown below the main panel for each sample).

While the PBMC used in these experiments were from normal patients, it is likely that the results may be similar to that in the allergic nasal mucosa in which chronic B cell stimulation by T cells occurs. The decrease in AID expression observed over time in the PBMC system is likely to have resulted from anti-CD40 mediated maturation of the B cells to the non-AID expressing plasma cell type [Muramatsu *et al.*, 1999]. In contrast, migration of $CD19^+$ B cells into the nasal mucosa must occur.

It is therefore likely that if AID expression were detected in the allergic nasal mucosa as a consequence of the T cell population, it could not be used as a direct and accurate marker of recent, local CSR and SHM because persistence of the signal for at least fourteen days would have to be assumed. The presence of AID mRNA would however strengthen evidence suggesting that B cells present in the nasal mucosa of allergic

rhinitis patients exist in local microenvironment in which all the known prerequisites for CSR and SHM exist.

8.3 AID mRNA splice variants.

Experiments with both stimulated PBMC and also the RAMOS cell line (used as a positive control) generated bands indicative of multiple PCR products. In addition to the expected AID PCR product at 647 bp, a particularly distinct DNA band was evident at 950 bp and much fainter bands at approximately 550 bp and 1400 bp (*Fig.* 8.3.1).

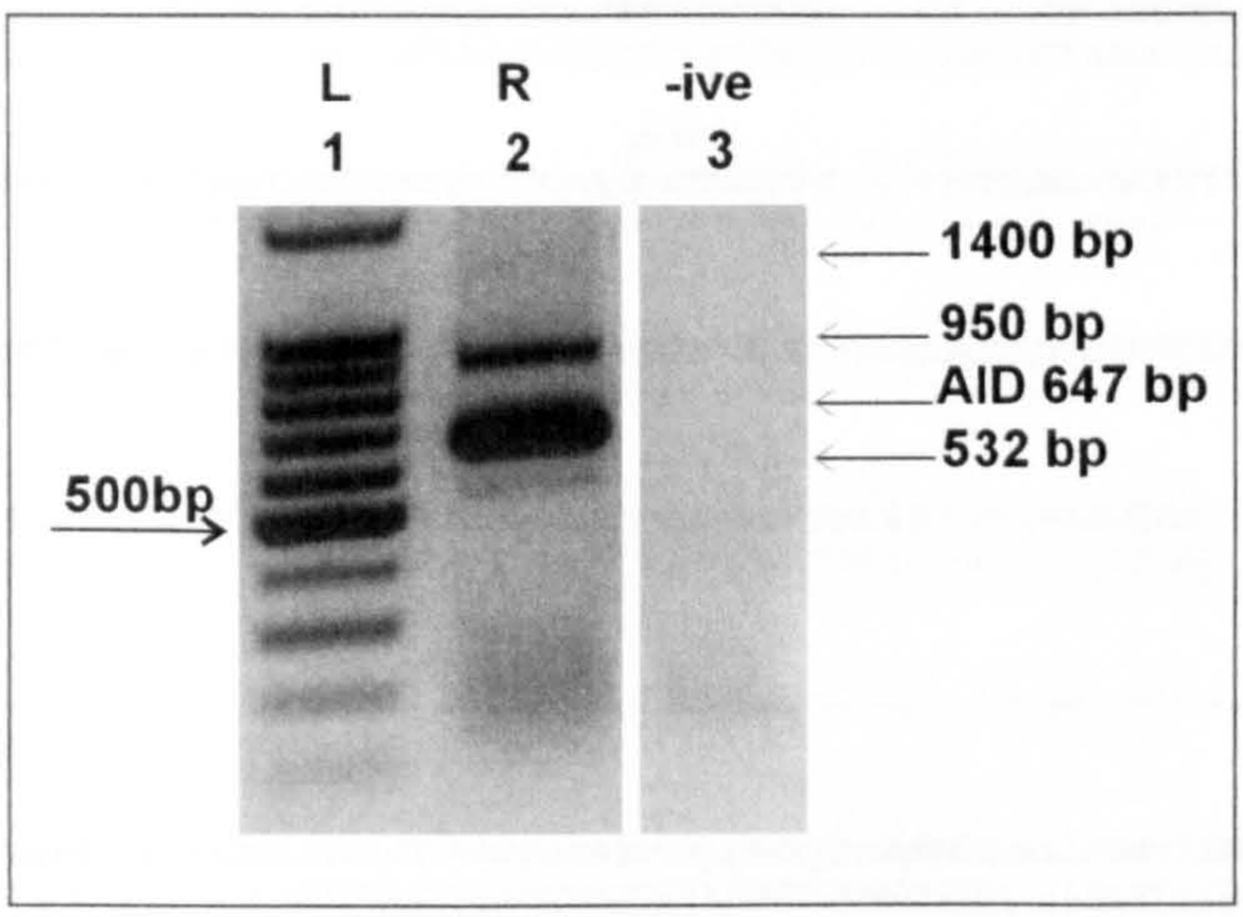


Fig. 8.3.1; Agarose gel electrophoresis of AID PCR products amplified from the RAMOS cell line demonstrating the presence of different mRNA splice variants. AID PCR products were subjected to agarose gel electrophoresis on a 1% agarose gel and visualised under UV. **Lane 1**; 100 bp ladder (L) (500 bp indicated). **Lane 2**; AID PCR products amplified from RAMOS were evident at approximately 532 bp, 647bp (as expected), 950 bp and 1400 bp. **Lane 3**; AID negative control (no cDNA).

Attempts were made to clone and sequence the unexpected 950 and 550 bp PCR products to establish their identity. Sequence analysis showed the approximately 550 bp PCR product to actually be 532 bp and while exhibiting homology with part of the AID sequence, was truncated as a consequence of the complete deletion of exon four (*Fig.* 8.3.2). This resulted in the consequent introduction of a stop codon in the amino acid sequence at the 5' end of exon five. Although the cytidine deaminase motif, thought to be the active site is encoded by exon three [Muto *et al.*, 2000], the conformational change to the AID protein as a result of the deletion of exon four and five must be so substantial as to render this variant inactive. This is particularly likely

given the detrimental effect of mutations observed in exon four of AID in patients suffering from HIGM2 [Revy *et al.*, 2000].

Fig. 8.3.2; Alignment of the 532 bp AID splice variant with the full-length AID sequence and the generation of a truncated amino acid sequence. **A)** The truncated 532 bp AID PCR product RT-PCR amplified from the RAMOS cell line was aligned with the full-length 647 bp AID sequence (accession no. AB040431). Primer regions are underlined. **B)** Translation of the 532 bp splice variant in reading frame two demonstrated the effect of truncating the amino acid sequence by the creation of a premature stop codon (*). The positions of exons 1-5 in the coding region only are marked on the full-length sequence in A) and B).

Unfortunately, the identity of the 950 bp band could not be determined in the time available, as the DNA appeared to be spliced to the correct size of 647 bp when cloned into the readily available strains of *E. coli*. It would seem sensible to assume however that the product contains intronic sequence. Within the PCR amplified region, only the

intron between exons three and four is of appropriate size (294 bp), making it a likely candidate for the additional sequence included within the 950 bp PCR product. The weak PCR product at approximately 1400 bp was also not investigated because of time constraints, but it would appear likely that it might correspond to a splice variant in which both the intron between exons three and four (294 bp) and also between exon four and five (470 bp) had not been excised.

8.4 Local expression of AID mRNA transcripts in the nasal mucosa of allergic rhinitis patients compared to normal subjects.

As discussed in the introduction, it was important to determine whether AID mRNA expression occurred in the nasal mucosa of allergic rhinitis patients as its presence is crucial at sites of CSR and SHM [Muramatsu *et al.*, 1999], [Muto *et al.*, 2000], [Fagarasan *et al.*, 2001]. Conventional PCR amplification of AID (see section 3.2.24) was attempted from the cDNA of a range of nasal biopsy samples, including the cohort of eleven patients detailed in chapters 4 and 6 (where the quantity of RNA remaining allowed). From only one of these samples were AID PCR products detected; in the nasal biopsy from allergic rhinitis patient DR20 (see section 3.2.2 for patient details), full-length AID mRNA was clearly and repeatedly amplified (*Fig.* 8.4.1). The identity of the PCR product was confirmed to be AID by sequence analysis (data not shown).

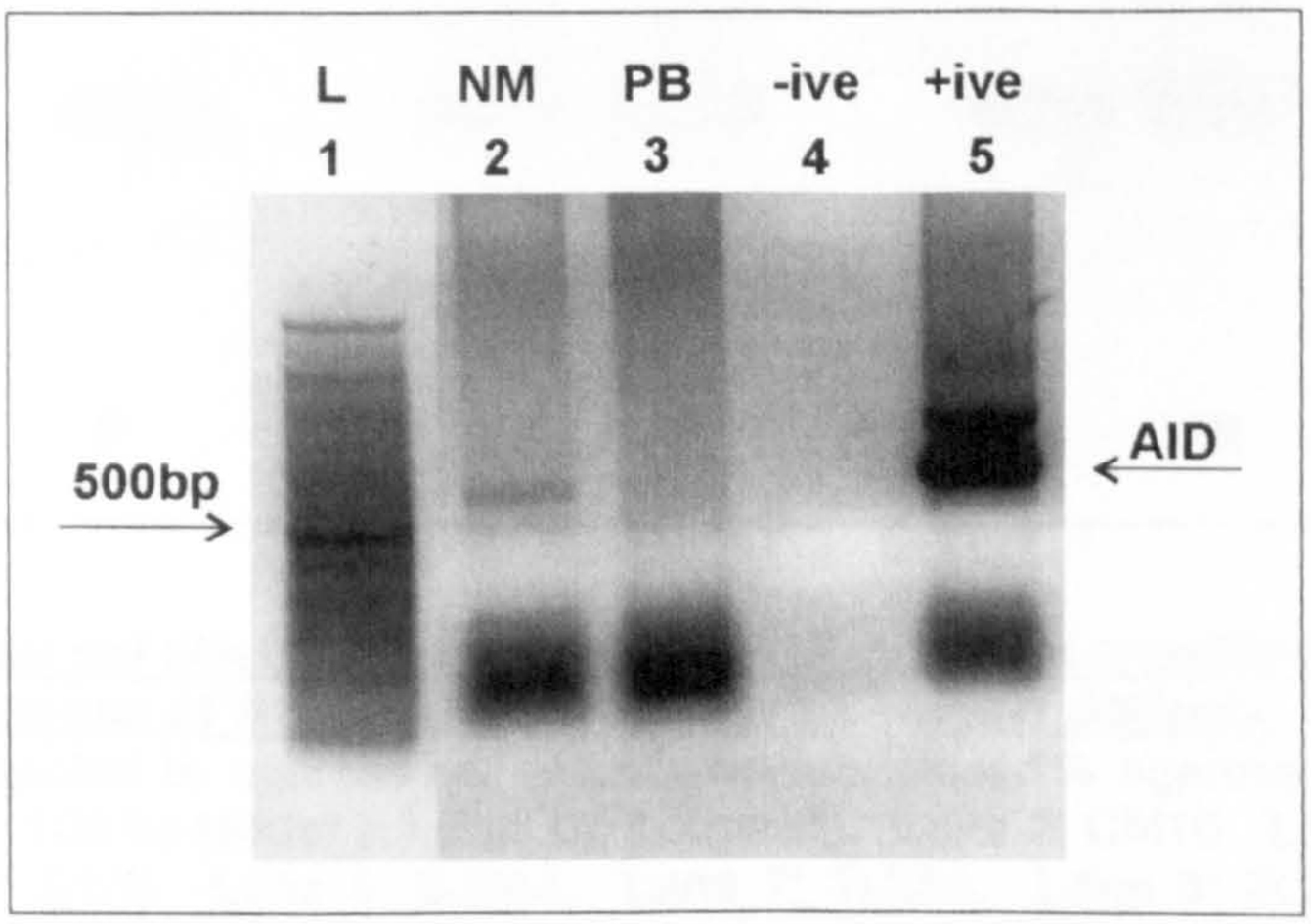


Fig. 8.4.1; Agarose gel electrophoresis of AID PCR products amplified from the nasal mucosa of allergic rhinitis patient DR20. AID PCR products (647 bp) were subjected to agarose gel electrophoresis on a 1% agarose gel and visualised under UV. **Lane 1**; 100 bp ladder (L) (500 bp indicated). **Lane 2**; DR20 nasal biopsy sample (NM). **Lane 3**; DR20 PBMC sample (PB). **Lane 4**; AID negative control (no cDNA). Lane 5; AID positive control (RAMOS cell line).

As it was possible that the limited sensitivity of the PCR prevented the detection of AID in the nasal biopsy samples, the PCR protocol was modified to utilise a nested approach (see section 3.2.24 for details). When AID mRNA expression in the nasal biopsies samples was re-analysed in this manner, PCR products were amplified from many of the samples. Preliminary results suggested that this nested PCR increased the sensitivity such that AID mRNA was evident in some, but not all, unstimulated normal and allergic PBMC samples (data not shown). However, presumably as a consequence of the greater sensitivity of the PCR, results from each sample were not always consistent when experiments were repeated. Therefore, only samples from which a PCR product was obtained in at least two out of four repeat experiments was classified as positive.

Of the seven allergic rhinitis patients in which sufficient nasal biopsy sample remained for repeated analysis, AID PCR products were definitely amplified from five (*Fig.* 8.4.2). These results were confirmed by Southern blot analysis using a probe that spanned the junction of exon three and four (data not shown).

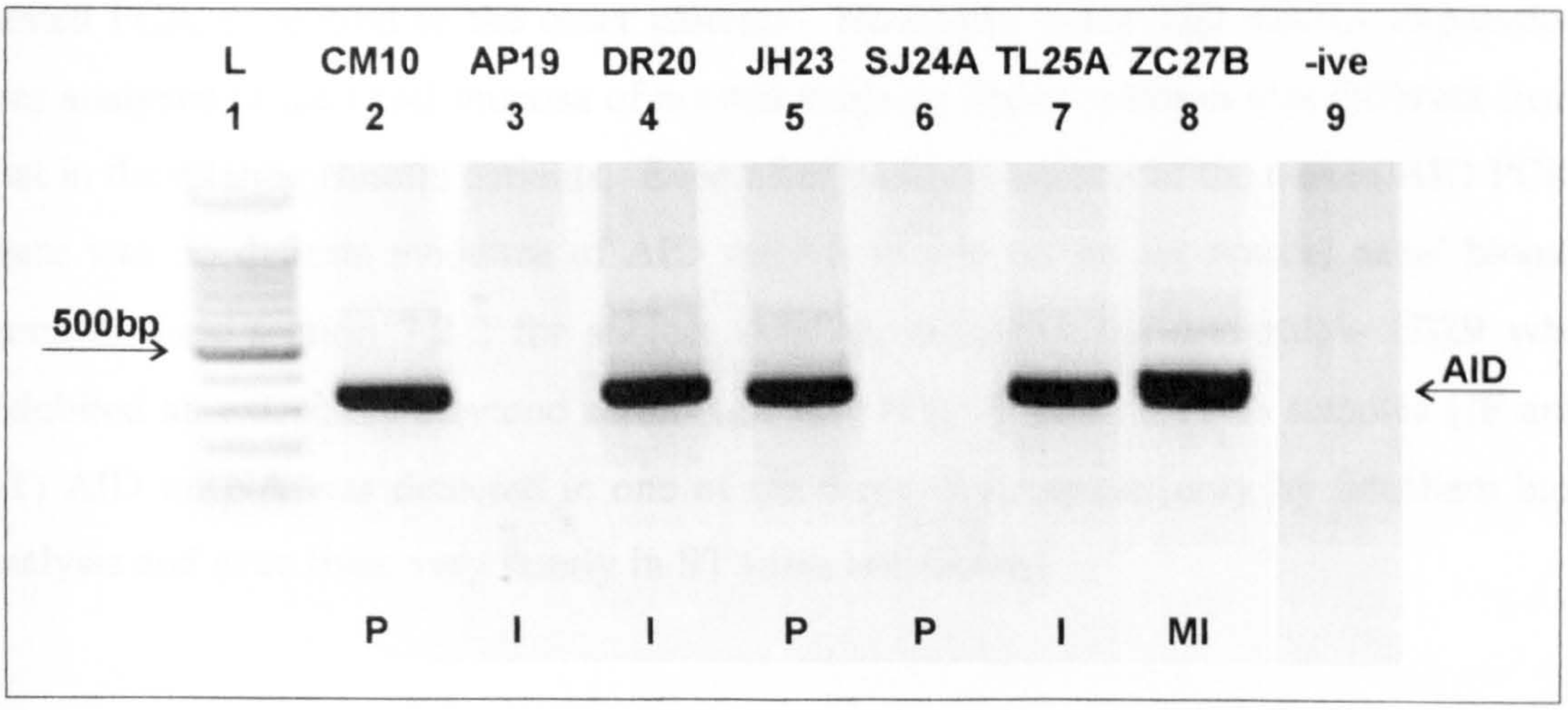


Fig. 8.4.2; Agarose gel electrophoresis of AID PCR products amplified by a nested PCR from the nasal mucosa of five out of seven allergic rhinitis patients. AID PCR products (335 bp) were subjected to agarose gel electrophoresis on a 1% agarose gel and visualised under UV. **Lane 1**; 100 bp ladder (L) (500 bp indicated). **Lane 2**; CM10. **Lane 3**; AP19. **Lane 4**; DR20. **Lane 5**; JH23. **Lane 6**; SJ24A. **Lane 7**; TL25A. **Lane 8**; ZC27B. **Lane 9**; AID negative control (no cDNA). P denotes a biopsy taken from a patient with perennial allergies. I denotes a biopsy taken in-season from a patient allergic to grass pollen and MI a monoallergic grass pollen allergic.

Interestingly, expression of AID mRNA was detected from the nasal mucosa of CM10, from which no related B cell clones were detected but was not detected in the nasal mucosa of AP19, from which evidence of related B cell clones was detected (chapter 4). In three of the seven patients in which local AID mRNA expression was analysed, multiple biopsies were taken (two half biopsies from SJ24, and double biopsies from both TL25 and ZC27). No AID mRNA could be definitely detected in either half of the biopsy from SJ24 (A or B), even though a family of clonally related B cells were detected from SJ24A (see chapter 6). In both TL25 and ZC27 however, although the double biopsies were adjacent pieces of tissue, different results were obtained; AID mRNA was clearly evident in TL25A and ZC27B, but not in TL25B and ZC27A (data not shown, but also confirmed by Southern blot analysis).

All of the biopsies detailed in *Fig.* 8.4.2 were either taken from grass pollen allergics in the grass pollen season, or from perennial allergic rhinitis sufferers. There was no correlation between AID mRNA expression and the season in which the tissue samples were taken, or whether the patients were monoallergic or multiallergic. Nor was there any apparent difference in DR20, in whom AID expression was evident without the nested PCR, compared to the other patients. However, when AID mRNA expression was analysed in the nasal mucosa of normal subjects, the expression was different from that in the allergic rhinitis patients. Even after multiple repeats of the nested AID PCR, there was no definite evidence of AID mRNA in any of the six normal nasal biopsy samples, (see section 3.2.2 for subject details), including the non-atopic GJ29 who exhibited an extremely elevated serum IgE titre (*Fig.* 8.4.3). In two samples (JF and ST) AID mRNA was detected in one of the three PCR repeats only by Southern blot analysis and even then, very faintly in ST (data not shown).

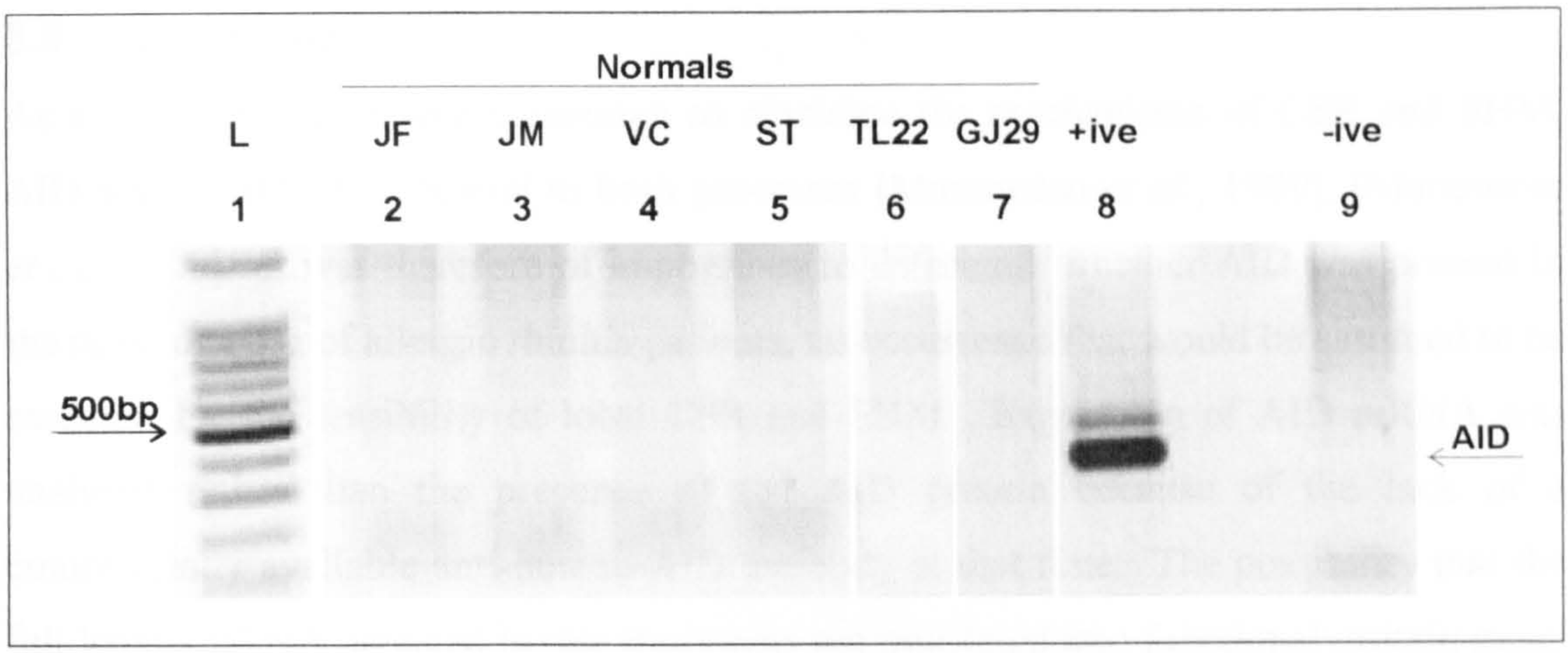


Fig. 8.4.3; Agarose gel electrophoresis of AID PCR products amplified by a nested PCR from the nasal mucosa of six normal subjects. AID PCR products (335 bp) amplified from nasal biopsy samples were subjected to agarose gel electrophoresis on a 1% agarose gel and visualised under UV. **Lane 1**; 100 bp ladder (L), (500 bp indicated). **Lane 2**; subject JF. **Lane 3**; subject JM. **Lane 4**; subject VC. **Lane 5**; subject ST. **Lane 6**; subject TL22. **Lane 7**; subject GJ29. **Lane 8**; AID positive control (RAMOS cell line). **Lane 9**; AID negative control (no cDNA).

In order to determine whether expression of AID could be induced in the nasal mucosa, nasal biopsies from four normal subjects were incubated *in vitro* for six days with IL-4 and anti-CD40. No evidence of AID expression was detected by RT-PCR from any of these samples, although GAPDH could be successfully amplified from each (data not shown). As the conditions for *in vitro* incubation of nasal biopsies had not been thoroughly optimised (a lengthy experiment currently being carried out in our group), a further experiment was designed in which *in vivo* allergen challenge with grass pollen extract was administered to six grass-pollen allergic rhinitis patients, outside of the grass pollen season. Nasal biopsies were taken five, six or seven days after allergen challenge. However, while GAPDH transcripts could again be amplified successfully from the samples, there was no evidence of AID expression, even by amplification with the nested PCR, although as no SCT's could be detected either (P. Takhar, personal communication) it is quite possible that the challenges were again unsuccessful for a technical reason.

8.5 Discussion.

As a consequence of recent research to elucidate the mechanisms of CSR and SHM, AID was identified as central to both processes [Muramatsu *et al.*, 1999], [Muramatsu *et al.*, 2000]. It was therefore of importance to determine whether AID was present in the nasal mucosa of allergic rhinitis patients, an occurrence that would be assumed to be essential for the feasibility of local CSR and SHM. Expression of AID mRNA was analysed rather than the presence of the AID protein because of the lack of a commercially available anti-human AID antibody at that time. The possibility that the full length mRNA detected in this study was not translated into functional protein must, while unlikely, be acknowledged.

Initial investigations were carried out to determine the timescale of the induction and maintenance of AID mRNA expression in the presence of IL-4 and anti-CD40, necessary not only for the induction of AID but also for CSR to IgE [Revy *et al.*, 2000], [Armitage *et al.*, 1993]. These experiments were carried out *in vitro* using normal PBMC and supported previously findings suggesting that AID mRNA expression was strongly expressed after incubation for five (or six) days and was still detectable after eight and twelve days [Revy *et al.*, 2000]. The results in this study more specifically determined that AID mRNA expression peaked after seven days of incubation and that while minimal AID expression was evident after fourteen days, it was much reduced by day ten.

While the initial up-regulation in the expression of AID mRNA coincided with the detection of SCTs, it was apparent that SCTs were the most accurate marker of recent CSR, as AID expression was maintained for a longer period of time (SCTs only being detected at days 6 and 7) (P. Takhar, unpublished observations). Results from the *in vitro* system implied however that in an environment in which IL-4 and anti-CD40 signals were plentiful (such as the allergic mucosa) [Ying *et al.*, 1994], AID expression might be stimulated, or previous stimulation of AID might be maintained. Indeed, recent research has demonstrated that in humans, IL-4 is sufficient to induce AID expression in both primary B cells and B cell lines, although in combination with CD40L, such expression was even further increased [Zhou *et al.*, 2003]. Expression of AID may only have been limited in the *in vitro* PBMC system by the differentiation of $CD19^+$ B cells into plasma cells. It would be interesting to determine if, as would be

expected, stimulation of allergic PBMC with IL-4 and anti-CD40 results in the same AID mRNA expression profile within a time-course experiment.

It was noted with interest that AID mRNA was detected for the first time in the allergic nasal mucosa, supporting the concept of local CSR and SHM. When RT-PCR was employed to amplify AID mRNA from nasal biopsy samples, initial results suggested the presence of AID in only one sample (DR20). When a nested PCR was employed however, it was evident that AID mRNA was present in the nasal mucosa of five out of seven allergic rhinitis patients at a lower, but indisputable level. In DR20 it is possible that the strong AID mRNA expression, evident even without nested PCR amplification, was a consequence of cellular stimulation five to seven days previously and that the B cells(s) were at the peak of their AID mRNA expression and therefore again a consequence of sampling.

This may mean that AID mRNA expression by nasal mucosal B cells was initially induced in the lymphoid tissue, but that migration of the B cell(s) to the nasal mucosa enabled the expression to be maintained as a consequence of the local IL-4 rich environment. It is also possible that the local microenvironment alone is sufficient to induce AID expression in B cells in the nasal mucosa. More research to determine the length of time of stimulation with IL-4 and anti-CD40 necessary to initiate and maintain AID mRNA expression needs to be carried out to elucidate which of these is the most likely. Further work to investigate the expression of AID within a single cell, rather than a population of cells would also contribute to understanding this. Optimisation of the *in vitro* incubation of nasal biopsies (unsuccessful in this study) to determine both the length of time that AID mRNA expression is maintained in tissue and also whether AID expression can be stimulated *in vitro* by allergen-challenge will also be of importance.

As with the investigation of local SHM and the spacial distribution of related cells, the presence or absence of AID reinforced the concept of the nasal mucosa consisting of confined areas of activity. Definite evidence of AID mRNA expression was detected from one part of a double biopsy but not the other, in biopsies from patients TL25 and ZC27. Immunohistochemistry to detect AID in the nasal mucosa of allergic rhinitis patients would be of particular interest to determine both the presence of the AID

protein and also the distribution of AID expression across the inferior turbinate, especially in comparison with the distribution of $CD19^+$ B cell clusters (see chapter 6).

It was interesting that of the nasal biopsies from the two allergic rhinitis patients in which V_H-Cε expression had previously been analysed (CM10 and AP19 in chapter 4), AID expression was evident in CM10 from which no reliable evidence of related B cell clones was detected, but was absent from AP19 from which a family of related IgE B cell clones had been detected. This suggests again that sampling may be of key importance; if the same biopsy had been taken from CM10 a day later for example, evidence of local CSR and SHM in the form of clonally related B cells may have been detected. The lack of AID mRNA expression in AP19 may indicate that the related B cell clones resulted from earlier SHM and clonal expansion. Detection of the clones may even have been facilitated by their maturation into plasma cells and the consequent increase in V_H-Cε mRNA available for PCR amplification.

There was no apparent correlation between the time of year at which the allergic patients were biopsied and the presence or absence of AID mRNA expression. Furthermore, although no AID mRNA was amplified from the unsuccessful *in vivo* grass pollen challenge of out of season patients (as might have been expected if AID mRNA expression was maintained in the absence of allergen), future work should be carried out to determine whether expression is maintained throughout the year in seasonal allergic rhinitis patients.

No clear expression of AID mRNA could be detected from the nasal biopsies of six normal subjects. This correlated with the high IL-4 and T cell rich environment being peculiar to the allergic mucosa [Varney *et al.*, 1992], [Ying *et al.*, 1994] and necessary to induce (or possibly maintain) AID expression in nasal B cells. In two of the normal subjects faint AID expression, evident by Southern blot analysis was detected in one of the repeat experiments, but was not reproducible. It is possible, if genuine, that this expression was a consequence of residual low-level AID mRNA expression by a B cell that had been stimulated previously in the lymphoid tissue. It is also always possible that a 'normal' patient may be allergic to an allergen not included on the skin-prick test panel.

In the course of the experimental investigation into AID mRNA expression, it became evident that splice variants of AID were detected in PBMC and the RAMOS cell line (there was no apparent evidence of this in the nasal biopsy samples). Initially, there were no published reports of AID mRNA splice variants. However, in 2001 their existence was confirmed by a publication that also documented linkage between inherited AID polymorphisms and asthma [Noguchi *et al.*, 2001]. In the study presented in this chapter, a splice variant characterised by the complete deletion of exon four was detected (532 bp). Noguchi *et al.* also detected this variant and also concluded that it was unlikely to have any activity given the distortion of the protein by the deletion of exons four and five in the amino acid sequence. Furthermore, Noguchi *et al.* were able to confirm that the truncated transcript was a mRNA splice variant by comparison with their patients' genomic AID sequence [Noguchi *et al.*, 2001]. In addition they also described a splice variant in which just 30 bp were deleted from exon four, which was not isolated in this study.

Noguchi *et al.* did not however comment upon the suggested AID splice variants detected in this study at approximately 950 bp and 1400 bp, hypothesized to be a consequence of the ineffective removal of the introns between exon three and four, or three, four and five respectively. While it is interesting to note the apparent inefficiency of the splicing of AID mRNA, this data highlights the importance of ensuring that AID mRNA amplification products are from viable, full-length mRNA transcripts. Noguchi *et al.* determined that there was no association between the presence of the AID splice variants and the presence of an AID polymorphism that they detected in exon four (7888C/T). However, this polymorphism was demonstrated to have an association with asthma and raised total serum IgE levels. It may therefore be interesting in the future to sequence all the PCR products amplified from the nasal biopsy samples in this study to analyse the presence of the 7888C/T polymorphism and determine whether such an association also exists in allergic rhinitis.

Chapter 9

Final discussion

9.1 Final discussion.

Local production of allergen specific IgE protein had previously been shown to occur in the nasal mucosa of allergic rhinitis patients [Smurthwaite *et al*, 2001] and the presence of both IgE^+ $CD19^+$ B cells and also IgE^+ $CD138^+$ plasma B cells identified in the allergic nasal mucosa [KleinJan *et al.*, 2000]. In addition, local production of IgE was shown to occur even in biopsies taken from grass pollen allergic patients outside of the pollen season, suggesting that the allergic mucosa was continuously primed to respond to allergen with immediate hypersensitivity. Furthermore, the production of allergen specific IgE from the allergic nasal mucosa correlated more closely with the patient's skin-prick test results and medical history than with the allergen specific IgE detected in the patient's serum [Smurthwaite *et al.*, 2001], [L. Smurthwaite, personal communication].

Other researchers demonstrated that when biopsies were taken from grass pollen allergic rhinitis patients after challenge with grass pollen allergen out of season, exposure to allergen correlated with an induction of IL-4 mRNA and ε germline transcripts [Durham *et al.*, 1997]. An increased number of cells expressing IL-4 mRNA and ε germline transcripts was also observed in allergic asthmatic patients, compared to controls [Ying *et al.*, 2001]. This suggested that the local microenvironment of the allergic mucosa was such that local class switch recombination to IgE might occur locally, consistent with the need for an immediate response to allergen. Support for this hypothesis was provided by analysis of V_H-Cε sequences amplified from the lung mucosa of an allergic asthmatic, which, on the basis of sequences from clonally related B cells, suggested that in addition to local class switch recombination, local somatic hypermutation and clonal expansion of B cells also occurred [Snow *et al.*, 1999].

In the previous five chapters, research undertaken to understand the molecular mechanisms of allergic rhinitis has been presented. This work has encompassed investigation to determine whether local somatic hypermutation and clonal expansion, local class switch recombination and also the local production of activation-induced cytidine deaminase occurs in the allergic nasal mucosa. In addition, the distribution of clonally related B cells and preliminary work to investigate the geographical distribution of B cells in the nasal mucosa was carried out.

In chapter 4, analysis of V_H-Cε sequences RT-PCR amplified from the nasal mucosa and for comparison, the PBMC of seven allergic rhinitis patients was undertaken. Sequences were deemed to have resulted from clonally related IgE^+ B cell clones if they exhibited a clonal signature region that resulted from the same V_H-D-J_H recombination event in a shared progenitor cell. In addition, these sequences exhibited both shared and unique somatic mutations acquired in a step-wise fashion as a consequence of the diversification of the B cell clonal family. Three distinct families of clonally related IgE^+ B cell clones were isolated from the nasal mucosa of two of the seven allergic rhinitis patients. Each family of related B cell clones consisted of two or three closely related members.

In contrast, only one family of related B cell clones was isolated from the PBMC samples provided by the seven allergic rhinitis patients. This family comprised two members that were more distantly related than any observed in the nasal mucosa, differing by many more mutations. Sequence analysis also suggested that while identical sister clones were present in both the nasal mucosa and PBMC of each of three of the patients, in one patient a clone was identified in the PBMC, that appeared to have diverged at an earlier stage in the evolution of a family of IgE^+ B cell clones isolated from that patient's nasal mucosa.

These results suggested that successive cycles of somatic hypermutation and clonal expansion had indeed occurred locally in the allergic nasal mucosa of at least two of the seven allergic rhinitis patients, resulting in the presence of closely related sister B cell clones in close proximity within the inferior turbinate. The alternative, that the cells had migrated individually from the lymphoid tissue to the same 2.5 mm^3 of the nasal mucosa from which the biopsy was taken, seemed too unlikely to be feasible, particularly as later work confirmed that the B cell repertoire of the allergic nasal mucosa was diverse and that related B cells from the same clonal family did not appear to be spread throughout the tissue. No V_H-Cε sequences could be amplified from the nasal mucosa of a healthy, non-atopic subject who exhibited an extremely high level of total serum IgE, suggesting that the local occurrence of such IgE^+ B cells was a feature of the local microenvironment of the nose in allergic rhinitis and not simply a result of the elevated serum IgE levels observed in those patients.

In chapter 5, a RT-PCR approach was utilised to determine from the amplification of V_H-C_H sequences from B cells expressing IgM, IgA or IgG, whether evidence of local class switch recombination was present in the allergic nasal mucosa. In one patient, five IgA^+ B cell clones were identified from the nasal mucosa that were further members of an IgE^+ B cell clonal family, previously identified in the same nasal biopsy. This data strengthened previous observations that local somatic mutation had occurred, but importantly it also suggested that switching had occurred locally. Again, it was unlikely that related IgA and IgE B cells, resulting from class switch recombination in the lymphoid tissue would have migrated to exactly the same area of the nasal mucosa. This observation has subsequently been supported by the detection of local switch circle transcripts in the nasal mucosa of allergic rhinitis patients [Takhar *et al.*, manuscript in preparation]. Interestingly, while the direction of the switch event, from an upstream isotype to both IgE and IgA or from IgE to $IgA_{(2)}$ for example, was not determined, previous researchers had only isolated IgM and IgG sequences from B cells related to IgE^+ sister clones from the allergic lung mucosa [Snow *et al.*, 1999].

As the results of these experiments supported the hypothesis of local somatic hypermutation and class switch recombination in the nasal mucosa of allergic rhinitis patients, it was of obvious importance to identify whether activation-induced cytidine deaminase (AID) was also present locally (chapter 8), (see publication included in appendix E). AID has been shown to be required for both class switch recombination and somatic hypermutation [Muramatsu *et al.*, 1999], [Muramatsu *et al.*, 2000], [Revy *et al.*, 2000], [Fagarasan *et al.*, 2001]. Its presence would therefore be expected to be necessary to support such a hypothesis of local events in the allergic nasal mucosa. The presence of mRNA encoding the full length AID transcript was indeed detected in five of the seven allergic rhinitis patients analysed. Furthermore, expression of AID mRNA was not evident in the nasal mucosa of the majority of normal subjects. Therefore, not only does the presence of AID support the hypothesis of local class switch recombination and somatic hypermutation in the allergic nasal mucosa, but its apparent absence from the nasal mucosa of normal subjects implies that the environment of the normal nasal mucosa, at least in times of health, is not suitable for switching or somatic mutation, even though this would perhaps be a sensible attribute.

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In respect of both somatic hypermutation, class switch recombination and AID expression, only samples from some allergic rhinitis patients yielded positive results. As there was no apparent difference between patients from whom positive compared to negative results were obtained, it was suggested that the sampling of the inferior turbinate might be responsible. The consistency of the cellular content of the inferior turbinate has not been investigated and it was proposed that if clonally related B cells were present in small groups or clusters that such clusters might be randomly excluded from the biopsy of one patient, but included in another.

When V_H-Cε sequences were analysed from adjacent pieces of tissue (chapter 6) it was evident that there was both a wide repertoire of IgE^+ B cells in the allergic nasal mucosa and also that the related IgE^+ B cell clones appeared to be concentrated in a small geographical area, as there was no evidence either of related or identical sister clones being present in adjacent pieces of tissue, even when the two halves of a nasal biopsy were examined. Furthermore, preliminary IHC (chapter 6) identified clusters of $CD19^+$ B cells in the nasal mucosa for the first time, while IgE^+ $CD138^+$ plasma cells appeared to occur individually.

Detailed analysis of V_H-Cε sequences isolated from the allergic rhinitis patients also supported the concept of the allergic nasal mucosa as a unique microenvironment. There was a highly significant difference in the usage of V_H5 in the allergic nasal mucosa compared to both that expected on the basis of the normal, rearranged genomic repertoire of the PBMC and also, importantly, that observed in the allergic PBMC. Even this biased use of V_H5 was not observed in all patients, or even in adjacent biopsies from the same patient, again suggestive of extreme local differences in the B cell clonal composition of the inferior turbinate.

When the V_H5 sequences from the nasal mucosa of the allergic rhinitis patients were analysed in greater detail, they exhibited an unusual distribution of somatic mutations across the V_H region. There was evidence of four potential non-intrinsic hotspots of mutation in the V_H5 sequences, suggesting that the V_H5 sequences were targeted towards a restricted repertoire of antigens. Unusually however, three of the non-intrinsic hotspots of mutation were situated in FWR1. The fourth non-intrinsic hotspot occurred in CDR2. Significantly, these non-intrinsic hotspots had all been previously

identified in V_H5 sequences amplified from the PBMC of allergic asthmatic patients [Snow *et al.*, 1997].

When the R/S values in the CDRs and FWRs were compared between the V_H5 and non-V_H5 sequences, the FWRs were shown to be subject to a greater degree of mutation in the V_H5 sequences and the normally significant difference observed between the FWR and CDR R/S values was abolished. In combination, the V_H5 bias, the unusual non-intrinsic hotspots of mutation and the unusual R/S values in the V_H5 sequences, suggested that in some patients, antigen selection by a B cell superantigen had occurred in the allergic nasal mucosa, consistent with that concluded by previous researchers in the allergic lung mucosa [Snow *et al.*, 1999] and the spleen of an allergic asthmatic [Snow *et al.*, 1995]. However, results from this study suggested that the effect of the superantigen was locally confined, in contrast to asthma and allergic dermatitis, in which similar trends were identified in the patient's PBMC [Snow *et al.*, 1997], [Van der Stoep *et al.*, 1993]. In this study a further hypothesis was proposed, that the B cell superantigen was likely to interact with FWR1 of V_H5 and also possibly CDR2 and FWR3.

Therefore, while the data presented in this thesis is largely circumstantial, it strongly supports the hypothesis that local class switch recombination, somatic hypermutation and clonal expansion occur locally in allergic rhinitis patients. This would appear to be coordinated by the local activity of activation-induced cytidine deaminase and as a consequence of the unique microenvironment of the allergic nasal mucosa. Exposure to allergenic antigens and possibly a superantigen(s) predispose and exacerbate the symptoms of allergic disease. Understanding fully the molecular mechanisms responsible for such allergy is of vital importance for the progression of therapies which, as a consequence of this and others research may now be targeted in a local fashion, thus enabling a much wider range of drugs, to treat the bacterial source of a superantigen, or to inhibit AID for example, to be contemplated.

Further work is being carried out by a subsequent PhD student to try to elucidate many of the interesting questions that have arisen as a result of the research presented in this thesis. In particular, further work has been targeted towards understanding the cellular composition of the nasal mucosa, especially with respect to clusters of $CD19^+$ B cells.

A greater number of well-characterised allergic and normal mucosa samples need to be analysed. From these samples it will be important to determine how frequently such $CD19^+$ B cell clusters occur and whether they are associated with the allergic state. In addition, it will be important to optimise both the IgE^+ $CD19^+$ double staining protocol to determine the distribution of such cells in the nasal mucosa and also to identify whether other cell types such as T cells or dendritic cells, for example, are present within, or close to, the clusters of $CD19^+$ B cells. Whilst the presence of AID mRNA in the allergic nasal mucosa has been identified in this study, it would also be of interest to utilise immunohistochemistry to confirm the presence of the AID protein and to identify if it is expressed by B cells associated with particular areas within the mucosa, such as the $CD19^+$ B cell clusters. It will also be of importance to determine whether such $CD19^+$ B cell clusters are associated with local SHM, clonal expansion and CSR and whether they resemble germinal centre B cells.

The other major area of research to be pursued further, is to identify the significance of the increased V_H5 use by B cells within the allergic nasal mucosa. Initially, work has progressed to study somatic mutations acquired in out-of-frame V_H5 sequences from normal subjects. As antigen selection can have placed no pressure upon the acquisition of these mutations, this should provide a more comprehensive picture of the intrinsic pattern of somatic mutation acquired by V_H5 sequences and is therefore of importance in confirming whether the non-intrinsic hotspots of mutation identified in chapter 7 are indeed likely to be antigen dependent. The use of V_H5 by other antibody isotypes in the allergic nasal mucosa is also to be investigated.

Future work has also been directed towards identifying the putative superantigen, proposed on the basis both of the increased use of V_H5 in the allergic nasal mucosa and the unusual non-intrinsic mutations that were identified from those sequences. It is hoped that this may be achieved by the amplification, cloning and expression of Fabs, isolated from singleV_H5 IgE^+ B cells, FACS sorted from the allergic inferior turbinate. To date, there are no crystal structures of any V_H5 molecule. In the interim, molecular modelling may enable the positions of the potential non-intrinsic mutations to be studied, but ultimately, it is hoped that this work may generate a crystal structure of V_H5. It is also anticipated that screening of these V_H5 Fabs may enable the identification of a V_H5 superantigen and crystallisation of a V_H5-antigen complex(es).

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Appendix A – DNA polymerase error rates.

In chapter 4 the difference in the experimental error rate when *Taq* and *Pfu* DNA polymerase were used was compared. The experimental error rates of *Taq* DNA polymerase (*Fig.* A.1) and *Pfu* DNA polymerase (*Fig.* A.2) were calculated as the number of mutations / number of base pairs amplified / number of cycles of PCR amplification. This was determined from analysis of the mutations introduced into Cε amplified in the V_H-Cε RT-PCR. The contribution of errors from other sources such as the reverse transcriptase step were not analysed separately from this overall error rate.

```
   CE1 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC 59 bp
  B5C2 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B5C3 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B5C4 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B5C5 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B5C6 CCTCCACACAGAGCCCATCCGTCCTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B5C7 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B5C8 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B5C9 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 B5C10 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 B5C11 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 B5C12 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 B5C13 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 B5C14 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 B5C15 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 B5C16 CCTTCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 B5C17 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 B5C18 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 B5C19 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 B5C20 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  P5C1 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  P5C2 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  P5C5 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  P5C6 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  P5C9 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACGTTCCCTCC
 P5C10 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 P5C11 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 P5C12 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 P5C15 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 P5C17 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 P5C18 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAGAAACATTCCCTCC
 P5C19 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
 P5C21 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
```

Fig. A.1; Alignment of Cε sequences amplified by V_H-Cε RT-PCR amplification with Taq DNA polymerase. Thirty-two Cε sequences amplified from the nasal biopsy and PBMC samples of patient SL5 by *Taq* DNA polymerase are aligned to the germline Cε sequence at the top of the figure. Sequences highlighted in red include a mutation from this germline Cε sequence and were used to calculate the experimental error rate.

Four mutations were evident in the 1888 bp of Cε sequence amplified by *Taq* DNA polymerase, an error rate of;

no. of mutations / no. of base pairs amplified / no. of cycles of PCR amplification

$(4 / 1888) / 60 = 3.53 \times 10^{-5}$ mut bp^{-1} dup^{-1}

This equates to one mutation being erroneously incorporated in experimental conditions including *Taq* DNA polymerase in approximately every 2 V_H regions analysed (each V_H region being approximately 282 bp).

```
     CE1 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC 59 bp
   B19C2 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B19C14 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
   B19C4 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B19C16 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
   B19C7 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
   B19C9 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B19C10 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
   B19C3 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
   B19C6 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B19C15 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B19C13 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  B19C12 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  P19C12 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  P19C18 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  P19C17 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
   P19C5 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
   P19C2 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  P19C24 CCTCCACATAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  P19C13 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
  P19C14 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
   P19C1 CCTCCACACAGAGCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCC
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Fig. A.2; Alignment of Cε sequences amplified by V_H-Cε RT-PCR amplification with Pfu DNA polymerase. The thirty-two Cε sequences amplified from the nasal biopsy and PBMC samples of seven patients (CD6, JB7, CM10, HD14, SO16, HD17 and AP19) by *Pfu* DNA polymerase are aligned to the germline Cε sequence at the top of the figure. Sequences highlighted in red include a mutation from this germline Cε sequence and were used to calculate the experimental error rate.

Two mutations were evident in the 9440 bp of Cε sequence amplified by *Pfu* DNA polymerase, an error rate of;

no. of mutations / no. of base pairs amplified / no. of cycles of PCR amplification
$(2 / (9440) / 60 = 3.53 \times 10^{-6}$ mut bp^{-1} dup^{-1}

This equates to one mutation being erroneously incorporated in experimental conditions including *Pfu* DNA polymerase in approximately every 17 V_H regions analysed (each V_H region being approximately 282 bp).

Appendix B – Chi-squared data.

In appendix B the data used to determine statistical significance according to the chi squared test is detailed.

The equation applied to the data was as follows;

$([\text{observed-expected}] - 0.5)^2$ / expected

This includes Yates' Correction for Continuity, which demands the subtraction of 0.5 from the [observed-expected]. This consequently results in a more conservative calculation of significance and is advisable when 'expected' values are less than 10.

Chi-squared analysis was used in chapter 7 to demonstrate the significance between the V_H gene usage in the allergic nasal mucosa compared to that expected. Expected values were based on the in-frame genomic rearrangements in normal PBMC observed by Brezinschek *et al.*, 2000.

Comparison of V_H5 usage in the nasal mucosa of allergic rhinitis patients and normal PBMC.

Raw data – observed number of sequences from eleven allergic rhinitis patients (detailed in Table 7.2.1) and an undefined number of normal patients [Brezinschek *et al.*, 2000].

	V_H5	**Non-V_H5**	**Total**
Allergic nasal mucosa	18	44	62
Normal PBMC	12	409	421
Total	30	453	483

Expected values - ((column total x row total) / grand total)

	V_H5	**Non-V_H5**
Allergic nasal mucosa	3.85	58.14
Normal PBMC	26.14	394.85

Chi-squared values – ([observed-expected] – 0.5)^2 / expected)

	V_H5	Non-V_H5
Allergic nasal mucosa	48.3955	3.2000
Normal PBMC	7.1174	0.4719

Total chi-squared value = 59.1848

The critical values for chi-squared distribution [Fentem, 1996] with one degree of freedom are;

p value	**0.99**	**0.975**	**0.95**	**0.9**	**0.1**	**0.05**	**0.025**	**0.01**	**0.005**
Chi-squared	0.000	0.001	0.004	0.016	2.706	3.841	5.024	6.635	7.879

The chi squared value therefore equates with a p value of 0.05 – 0.025

When $p < 0.05$ = the difference is significant, when $p < 0.01$ the difference is highly significant.

The difference between V_H5 gene usage in allergic rhinitis patients and normal PBMC is therefore highly significant.

Comparison of V_H5 gene usage in the nasal mucosa of allergic rhinitis patients and normal PBMC.

Raw data – observed number of sequences from seven allergic rhinitis patients (excluding the major V_H5 contributor CA30, as detailed in chapter 7) and an undefined number of normal patients [Brezinschek *et al.*, 2000].

	V_H5	Non-V_H5	Total
Allergic nasal mucosa	6	31	37
Normal PBMC	12	409	421
Total	18	440	458

Expected values - ((column total x row total) / grand total)

	V_H5	Non-V_H5
Allergic nasal mucosa	1.45	35.55
Normal PBMC	16.55	404.45

Chi-squared values – ([observed-expected] – 0.5)^2 / expected)

	V_H5	Non-V_H5
Allergic nasal mucosa	11.3121	0.4614
Normal PBMC	0.9911	0.0406

Total chi-squared value = 12.8052

The difference between V_H5 usage in the allergic rhinitis patients (excluding CA30) and normal PBMC is therefore still highly significant.

Comparison of V_H5 gene usage in allergic and normal PBMC.

Raw data – observed number of sequences from seven allergic rhinitis patients (detailed in Table 7.2.1) and an undefined number of normal patients [Brezinschek *et al.*, 2000].

	V_H5	**Non-V_H5**	**Total**
Allergic PBMC	4	46	50
Normal PBMC	12	409	421
Total	16	455	471

Expected values - ((column total x row total) / grand total)

	V_H5	**Non-V_H5**
Allergic PBMC	1.70	48.30
Normal PBMC	14.30	406.70

Chi-squared values – ([observed-expected] – 0.5)^2 / expected)

	V_H5	**non-V_H5**
Allergic PBMC	1.9059	0.0671
Normal PBMC	0.2266	0.0080

Total chi-squared value =2.2076

The difference between V_H5 usage in allergic and normal PBMC is not therefore significant.

Comparison of V_H5 gene usage in the nasal mucosa and PBMC of allergic rhinitis patients.

Raw data – observed number of sequences from the nasal mucosa of eleven allergic rhinitis patients (detailed in Table 7.2.1) and from the PBMC of seven allergic rhinitis patients.

	V_H5	**Non-V_H5**	**Total**
Allergic nasal mucosa	18	44	62
Allergic PBMC	4	46	50
Total	22	90	112

Expected values - ((column total x row total) / grand total)

	V_H5	**Non-V_H5**
Allergic nasal mucosa	12.18	49.82
Allergic PBMC	9.82	40.18

Chi-squared values – ([observed-expected] – 0.5)^2 / expected)

	V_H5	**Non-V_H5**
Allergic nasal mucosa	2.3237	0.5681
Allergic PBMC	2.8821	0.7044

Total chi-squared value = 6.4783

The difference between V_H5 usage in the allergic nasal mucosa and allergic PBMC is therefore of significance.

Comparison of V_H5 gene usage in the nasal mucosa and PBMC of allergic rhinitis patients.

Raw data – observed number of sequences from seven allergic rhinitis patients (excluding the major V_H5 contributor CA30, as detailed in chapter 7) and from the PBMC of seven allergic rhinitis patients.

	V_H5	Non-V_H5	Total
Allergic nasal mucosa	6	31	37
Allergic PBMC	4	46	50
Total	10	77	87

Expected values - ((column total x row total) / grand total)

	V_H5	Non-V_H5
Allergic nasal mucosa	4.25	32.75
Allergic PBMC	5.75	44.25

Chi-squared values – ([observed-expected] – 0.5)^2 / expected)

	V_H5	Non-V_H5
Allergic nasal mucosa	0.3676	0.0477
Allergic PBMC	0.2717	0.0353

Total chi-squared value = 0.7223

The difference between V_H5 usage in the allergic nasal mucosa and allergic PBMC (excluding CA30) is not therefore of significance.

Comparison of V_H3 usage in the nasal mucosa of allergic rhinitis patients and normal PBMC.

Raw data – observed number of sequences from eleven allergic rhinitis patients (detailed in Table 7.2.1) and an undefined number of normal patients [Brezinschek *et al.*, 2000].

	V_H3	Non-V_H3	Total
Allergic nasal mucosa	21	41	62
Normal PBMC	232	189	421
Total	253	230	483

Expected values - ((column total x row total) / grand total)

	V_H3	Non-V_H3
Allergic nasal mucosa	32.48	29.52
Normal PBMC	220.52	200.48

Chi-squared values – ([observed-expected] – 0.5)^2 / expected)

	V_H3	Non-V_H3
Allergic nasal mucosa	3.7118	4.0840
Normal PBMC	0.5467	0.6014

Total chi-squared value = 8.9439

The difference between V_H3 gene usage in allergic rhinitis patients and normal PBMC is therefore highly significant.

Comparison of V_H3 gene usage in allergic and normal PBMC.

Raw data – observed number of sequences from seven allergic rhinitis patients (detailed in Table 7.2.1) and an undefined number of normal patients [Brezinschek *et al.*, 2000].

	V_H3	**Non-V_H3**	**Total**
Allergic PBMC	25	25	50
Normal PBMC	232	189	421
Total	257	214	471

Expected values - ((column total x row total) / grand total)

	V_H3	**Non-V_H3**
Allergic PBMC	27.28	22.72
Normal PBMC	229.72	191.28

Chi-squared values – ([observed-expected] – 0.5)^2 / expected)

	V_H3	**non-V_H3**
Allergic PBMC	0.1161	0.1395
Normal PBMC	0.0138	0.0166

Total chi-squared value =0.286

The difference between V_H3 usage in allergic and normal PBMC is not therefore significant.

Comparison of V_H3 gene usage in the nasal mucosa and PBMC of allergic rhinitis patients.

Raw data – observed number of sequences from the nasal mucosa of eleven allergic rhinitis patients (detailed in Table 7.2.1) and from the PBMC of seven allergic rhinitis patients.

	V_H3	**Non-V_H3**	**Total**
Allergic nasal mucosa	21	41	62
Allergic PBMC	25	25	50
Total	46	66	112

Expected values - ((column total x row total) / grand total)

	V_H3	**Non-V_H3**
Allergic nasal mucosa	25.46	36.54
Allergic PBMC	20.54	29.46

Chi-squared values – ([observed-expected] – 0.5)^2 / expected)

	V_H3	**Non-V_H3**
Allergic nasal mucosa	0.6159	0.4292
Allergic PBMC	0.7635	0.5323

Total chi-squared value = 2.3409

The difference between V_H3 usage in the allergic nasal mucosa and allergic PBMC is not therefore significant.

Chi-squared analysis was also used to analyse the difference between the observed and expected R/S values in the FWRs compared to the CDRs in V_H5 and non-V_H5 sequences (chapter 7).

Comparison of R/S values in the CDR and FWR of V_H5 sequences from allergic rhinitis patients (seventeen sequences).

Raw data

	CDR	FWR	Total
Replacement mutations	76	146	222
Silent mutations	32	71	103
Total	108	217	325

Expected values - ((column total x row total) / grand total)

	CDR	FWR
Replacement mutations	73.77	148.23
Silent mutations	34.23	68.77

Chi-squared values – ([observed-expected] – 0.5)^2 / expected)

	CDR	FWR
Replacement mutations	0.0406	0.0202
Silent mutations	0.0874	0.0435

Total chi-squared value = 0.1917

The difference between the R/S values in the CDR and FWR of the V_H5 sequences from the allergic nasal mucosa is not therefore significant.

Comparison of R/S values in the CDR and FWR of non-V_H5 sequences from allergic rhinitis patients (nineteen sequences).

Raw data

	CDR	FWR	Total
Replacement mutations	83	107	190
Silent mutations	24	64	88
Total	107	171	278

Expected values - ((column total x row total) / grand total)

	CDR	FWR
Replacement mutations	73.13	116.87
Silent mutations	33.87	54.13

Chi-squared values – ([observed-expected] – $0.5)^2$ / expected)

	CDR	FWR
Replacement mutations	1.2006	0.7512
Silent mutations	2.5922	1.6220

Total chi-squared value = 6.166

The difference between the R/S values in the CDR and FWR of the non-V_H5 sequences from the allergic nasal mucosa is therefore significant.

Appendix C – Directionality of somatic hypermutation.

In chapter 7 the expected direction of somatic mutation, predicted on the basis of the intrinsic bias of SHM, was calculated and compared to the number of mutations observed at hotspots in the V_H5 and non-V_H5 data sets. A departure from the expected values indicated that the mutation was likely to be a non-intrinsic hotspot, selected by antigen. The expected direction of mutation was predicted on the basis of the preferences of SHM that were determined by Betz *et al.*, 1993b, detailed below.

To	T	C	A	G
From				
T	-	0.52	0.31	0.17
C	0.74	-	0.09	0.17
A	0.29	0.19	-	0.52
G	0.09	0.32	0.59	-

Table C.1; The substitution preferences of somatic hypermutation [Betz et al., 1993b].

Therefore, if ten mutations occurred at a hotspot substituting A, the predicted direction of mutation would be;

A→C	2	(0.19 x 10)
A→G	5	(0.52 x 10)
A→T	3	(0.29 x 10)

Appendix D – R/S values.

In chapter 7, the distribution of replacement and silent mutations were analysed by comparing the R/S values in different data sets. Replacement mutations generated a change in the amino acid, while silent mutations had no effect on the amino acid that was encoded. This meant that the number of individual nucleotide mutated within a codon could not be taken into account. Only one sequence was used to represent a family of related B cells clones, however each mutation in any clone from that family was included once in that representative sequence.

Initially the overall R/S values in the CDRs and FWRs were compared between the V_H5 and non-V_H5 sequences (Table 7.4.1 details both the final R/S values and the raw data). These overall R/S values were calculated by pooling the total number of replacement and number of silent mutations observed in the sequences comprising each data set

e.g. the overall R/S value in the V_H5 CDR =

total no. replacement / total no. silent mutations in the CDRs of the V_H5 sequences

76 / 32 = 2.06

The overall R/S value of the combined FWRs and also the combined CDRs was also calculated for each individual sequence (Table 7.4.2). The raw data and final R/S values for each of these sequences is detailed below in Tables D.1 and D.2.

In instances where there were zero silent mutations, but there were replacement mutations, the R/S value was preceded by >. Where there were zero replacement mutations, but there were silent mutations, the R/S value was preceded by <. While not strictly accurate this was determined to be the best way in which to distinguish such values. This was only an issue when individual sequences were examined and did not affect the overall interpretation of any of the results.

	CDR			FWR		
	R	S	R/S	R	S	R/S
HD14 C7	6	1	6.0	8	4	2.0
SO16 C11	6	1	6.0	10	4	2.5
CA30A C12	6	0	>6.0	6	3	2.0
CA30A C11	6	1	6.0	12	5	2.4
CA30A C16	4	1	4.0	6	4	1.5
CA30A C22	5	1	5.0	6	12	0.5
CA30A C4b	4	0	>4.0	8	5	1.6
HD14 C12	1	4	0.3	5	3	1.7
TL25B C8	4	2	2.0	4	2	2.0
CA30A C8	7	2	3.5	6	2	3.0
CA30A C17	6	2	3.0	10	4	2.5
CA30A C3	3	5	0.6	9	10	0.9
CA30A C20	5	3	1.7	12	5	2.4
HD14 C19	3	2	1.5	6	0	>6.0
AP19 C2	2	4	0.5	11	3	3.7
CA30A C5	4	0	>4.0	5	1	5.0
CA30A C3b	4	3	1.3	20	4	5.0

Table D.1; The raw numbers of replacement (R) and silent (S) mutations and the R/S value in the CDR and FWR of each V_H5 sequence amplified from the allergic nasal mucosa (ordered to correspond with Table 7.4.2).

	CDR			FWR		
	R	S	R/S	R	S	R/S
CD6 C1	3	1	3.0	4	2	2.0
CD6C13	7	1	7.0	5	2	2.5
HD14 C2	5	0	>5.0	8	3	2.7
HD14 C4	8	2	4.0	8	8	1.0
HD14 C11	3	1	3.0	6	4	1.5
HD14 C16	5	1	5.0	5	5	1.0
SO16 C13	5	1	5.0	2	1	2.0
CA30B C7	5	0	>5.0	5	3	1.7
TL25A C17	7	2	3.5	5	3	1.7
CD6 C12	2	2	1.0	7	3	2.3
CD6 C2	3	2	1.5	7	5	1.4
HD14 C9	7	3	2.3	7	6	1.2
AP19C16	2	2	1.0	0	1	<1.0
CA30B C8	1	0	>1.0	3	5	0.6
CA30B C19	5	3	1.7	10	4	2.5
CD6 C5	3	1	3.0	5	1	5.0
AP19 C3	2	0	>2.0	4	1	4.0
AP19 C6	3	2	1.5	7	2	3.5

Table D.2; The raw numbers of replacement (R) and silent (S) mutations and the R/S value in the CDR and FWR of each non-V_H5 sequence amplified from the allergic nasal mucosa (ordered to correspond with Table 7.4.2).

The R/S values of each of the FWRs, 1, 2 and 3 were also determined for each individual V_H5 sequence. The overall V_H5 FWR1, 2 and 3 R/S values were additionally calculated for the combined sequences (Table D.3).

	FWR1			FWR2			FWR3		
	R	S	R/S	R	S	R/S	R	S	R/S
HD14 C7	3	1	3.0	1	1	1.0	4	2	2.0
SO16 C11	4	1	4.0	0	2	<2.0	6	0	>6.0
CA30A C12	2	2	1.0	0	0	0.0	4	1	4.0
CA30A C11	5	1	5.0	1	1	1.0	6	3	2.0
CA30A C16	1	1	1.0	0	2	<2.0	5	1	5.0
CA30A C22	1	4	0.25	2	2	1.0	3	6	0.5
CA30A C4b	3	3	1.0	2	1	2.0	3	1	3.0
HD14 C12	2	1	2.0	0	0	0.0	1	1	1.0
TL25B C8	2	0	>2.0	1	1	1.0	1	1	1.0
CA30A C8	4	1	4.0	0	0	0.0	2	1	2.0
CA30A C17	2	2	1.0	2	1	2.0	6	1	6.0
CA30A C3	3	4	0.8	0	2	<2.0	6	4	1.5
CA30A C20	5	1	5.0	2	1	2.0	5	3	1.7
HD14 C19	2	0	>2.0	0	0	0.0	4	0	>4.0
AP19 C2	4	1	4.0	2	2	1.0	5	0	>5.0
CA30A C5	4	1	4.0	2	1	2.0	2	0	>2.0
CA30A C3b	7	1	7.0	3	0	>3.0	10	3	3.3
Combined	54	25	2.16	17	16	1.06	73	28	2.61

Table D.3; The raw numbers of replacement (R) and silent (S) mutations and the R/S value in FWR1, 2 and 3 of each V_H5 sequence and the combined sequences, amplified from the allergic nasal mucosa (ordered to correspond with Table 7.4.2).

The FWR3 R/S values of the non-V_H5 sequences was also calculated (Table D.4).

	FWR3		
	R	S	R/S
CD6 C1	4	2	2.0
CD6C13	4	1	4.0
HD14 C2	6	2	3.0
HD14 C4	6	7	0.9
HD14 C11	1	3	0.3
HD14 C16	2	3	0.7
SO16 C13	1	1	1.0
CA30B C7	2	3	0.7
TL25A C17	4	3	1.3
CD6 C12	6	2	3.0
CD6 C2	4	2	2.0
HD14 C9	5	4	1.3
AP19C16	0	1	<1.0
CA30B C8	2	2	1.0
CA30B C19	6	2	3.0
CD6 C5	5	1	5.0
AP19 C3	3	1	3.0
AP19 C6	5	2	2.5
Combined	69	44	1.57

Table D.4; The raw numbers of replacement (R) and silent (S) mutations and the R/S value in FWR3 of each non-V_H5 sequence and the combined sequences, amplified from the allergic nasal mucosa (ordered to correspond with Table 7.4.2).

Appendix E – Related publication.

Publication in press:

Coker, H. A., Durham, S. R., Gould, H. J. Local somatic hypermutation and class switch recombination in the nasal mucosa of allergic rhinitis patients. 2003, *J. Immunol.*, 171, 5602-5610.

Local Somatic Hypermutation and Class Switch Recombination in the Nasal Mucosa of Allergic Rhinitis Patients

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Footnotes

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[3] **Abbreviations used in this paper:** CSR, class switch recombination; SHM, somatic hypermutation; CDR, complementarity determining region; FWR, framework region; AID, activation-induced cytidine deaminase; RAST, radioallergosorbent test.

Abstract

Immunoglobulin E is produced by nasal B cells in response to allergen. We have analyzed IgE V_H region sequences expressed in the nasal mucosa of patients suffering from allergic rhinitis. V_H region sequences were amplified by RT-PCR from IgE^+ B cells from nasal biopsies. In two out of six patients, sequence analysis clearly demonstrated the presence of closely related IgE^+ B cell clones; cells displaying identical 'signature regions' across CDR3/FWR4, indicating a common clonal ancestry, but a mixture of shared and diverse somatic mutations across the V_H region. Furthermore, in one of the two patients exhibiting related IgE^+ B cell clones, five IgA^+ B cell clones, related to the IgE^+ B cell family were also isolated from the patient's nasal mucosa. This evidence, combined with the local expression of mRNA transcripts encoding activation-induced cytidine deaminase, suggests that local somatic hypermutation, clonal expansion and class switch recombination occur within the nasal mucosa of allergic rhinitics. The presence of related B cells in the nasal mucosa does not appear to result from the random migration of IgE^+ cells from the systemic pool, as analysis of a non-atopic subject with highly elevated serum IgE did not exhibit any detectable V_H-Cε transcripts in the nasal mucosa. We have provided evidence that suggests for the first time that the nasal mucosa of allergic rhinitics is an active site for local somatic hypermutation, clonal expansion and class switch recombination, making it of major significance for the targeting of future therapies.

Introduction

Allergic reactions occur mainly in the mucosal tissues of the respiratory tract, gut and skin. Susceptibility at these sites is due to the presence of mast cells bearing the high-affinity immunoglobulin E (IgE) receptor, FcεRI, sensitized by antibodies of the IgE class. Cross-linking of the receptors by allergen binding to IgE antibodies triggers immediate hypersensitivity. Mast cells in the nasal mucosa of patients with allergic rhinitis are sensitized by IgE antibodies that are produced locally by resident plasma cells (1). In an allergic individual, local IgE production persists for long periods in the absence of allergen, enabling an immediate response upon re-exposure to allergen. However, little is known about the history of the IgE-producing B cells, in particular when and where the precursor cells underwent class switch recombination (CSR)[3] to IgE (involving rearrangement of the constant region genes encoding the various antibody classes, e.g. to Cε) and affinity maturation by somatic hypermutation (SHM).

Both SHM and CSR are stimulated by antigen in the germinal centres of lymphoid tissue (2), but it is becoming increasingly apparent that these processes may also occur locally at sites of chronic or recurrent antigen stimulation as has been clearly demonstrated for rheumatoid arthritis (3, 4). In the local mucosal environment, signals to initiate SHM and CSR are available (5, 6) as *in vitro* studies have demonstrated that, in the presence of antigen, T cells have the ability to induce SHM and CSR by the production of cytokines (such as IL-4) and also their interaction via CD40:CD40-ligand and CD80:CD28 with B cells (7). Evidence suggesting local CSR has been presented for IgA in the murine gut mucosa (8), for IgE in the human nasal mucosa of allergic rhinitics (9-11) and in the human lung mucosa in allergic asthma (12, 13). Evidence for local SHM has been presented for IgE in the lung mucosa of an allergic asthmatic patient (13). We have examined the evidence for local SHM and CSR in six patients with allergic rhinitis.

SHM results from the stepwise accumulation of predominantly single nucleotide substitutions into the V region DNA. This stepwise accumulation of mutations enables the genealogy of a B cell to be traced, relying on the unique complementarity determining region 3/framework region 4 (CDR3/FWR4) 'clonal signature' generated by the VDJ recombination of the progenitor cell. It is estimated that mutations are introduced at a rate of 10^{-4}-10^{-3} per base pair per generation (14). A single mutation may bring about as much as a ten-fold increase in antibody affinity (14, 15). Mutations are introduced at a high level across the CDRs but additionally the RGYW motif (where R=A or G, Y=C or T, W=A or T and G is mutated) or the reverse complement WRCY is a frequent target of mutation, particularly at the serine codons AGC and AGT (16). The molecular mechanism of SHM has not been fully elucidated, although errors are thought to be introduced by an activation-induced cytidine deaminase (AID) and error-prone polymerase dependent process (17-19). Several DNA polymerases have been suggested as candidates, including DNA polymerase η, κ, ι and ζ (20 - 23). AID has also been shown to be required for CSR (17, 18).

We present evidence generated by RT-PCR and DNA sequencing of V_H-Cε sequences from the nasal mucosa of allergic rhinitis patients. Our work is the first to demonstrate in two allergic rhinitis patients the clear presence of related IgE^+ B cell clones in the nasal mucosa, B cells that exhibit shared ancestry (judged by identical CDR3/FWR4 motifs) and both shared and diverse somatic mutations. In addition, detailed investigation enabled the detection of sequences from IgA^+ B cell clones from the nasal biopsy of one patient. These sequences exhibited shared ancestry and both shared and diverse somatic mutations

with the related IgE^+ B cell clones isolated from the same nasal biopsy sample. IgE V_H-C_H region amplification from the nasal mucosa of a healthy non-atopic subject with highly elevated serum IgE was negative, implying that the families of related clones seen in allergic patients were unlikely to have resulted from the random migration of IgE^+ B cells from a systemic pool.

Furthermore, RT-PCR analysis demonstrated the presence of mRNA encoding AID in the nasal mucosa in five out of seven allergic rhinitis patients, the first reported instance of local AID expression in humans. We propose that local somatic hypermutation, clonal expansion and class switch recombination take place in the nasal mucosa of allergic rhinitis patients. We suggest that this local activity is fundamental to the pathogenesis of allergic disease.

Materials and Methods

Samples from allergic rhinitis patients

Male and female donors between the ages of eighteen and fifty-five were recruited from the Royal Brompton Hospital Allergy Clinic or by advertisement in the local press. None had received immunotherapy and any medication was discontinued at least two weeks before nasal biopsy. Biopsies were performed at the Royal Brompton Hospital with the approval of the local Ethics Committee and the patients' written informed consent. The allergic status of the donors was assessed by medical history, skin-prick tests and serum allergen specific IgE (RAST). Six patients who exhibited a total IgE of over 200 IU/ml were selected for the study of V_H-Cε transcripts. Biopsies were taken according to a previous detailed protocol (24) in which a 2.5 mm^3 biopsy was randomly taken from the under surface of the inferior turbinate, behind the anterior insertion into the lateral wall of the nose and 8-10 cm distal to the nearest lymphoid tissue, Waldeyer's ring in the pharynx. The random nature of the biopsies prevented the sampling of any defined cell populations from within the inferior turbinate. Biopsies were placed in 1.5 ml Hank's balanced saline solution (GibcoBRL) and washed to remove blood before being transferred to a cryotube and snap frozen in liquid N_2 before being stored at –70°C.

25 ml blood was taken from each patient and PBMC isolated by Ficoll-Paque density gradient centrifugation. The PBMC pellet was snap-frozen and stored at –70°C. The samples were treated to extract RNA and manufacture cDNA as for the tissue samples, with the exception that 1 ml RNAWIZ buffer was added to the PBMC pellet (ca. 4×10^7 cells) and the cells resuspended by pipetting before dividing into four 250 µl aliquots, one of which was used for future molecular analysis.

Samples from the non-atopic subject

A healthy 55 year old male was recruited with no previous medical history of any allergic symptoms and a negative skin-prick test and RAST for all common allergens (a slightly raised RAST of 0.71 IU/ml (normal <0.35 IU/ml) was detected for *Aspergillus fumigatus*). No other abnormal medical conditions were reported although the subject's total serum IgE was 1834 IU/ml. A nasal biopsy and PBMC sample were taken according to the protocol above.

Amplification of GAPDH from the samples utilised the following PCR method; 5 µl of cDNA was added to a 50 µl PCR reaction, which included both a GAPDH forward and reverse primer each at 1 µM (GAPDH forward; 5'- ATTTGGTCGTATTGGGCGCCTGGTC-3', GAPDH reverse; 5'-TCATACTTCTCATTGTTCACACCCATG-3'), dNTP's at 0.25 mM and included 1.26 U *Pfu* DNA polymerase (Promega). The reaction was initially denatured at 95°C for 2 min, then subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 2 min, and extension at 72°C for 2 min before a final extension at 72°C for 10 min.

RNA extraction

Biopsies were transferred to an RNase-free tube and homogenized in 250 µl RNAWIZ buffer (Ambion) with 300 vigorous pulses using an Eppendorf homogeniser (Anachem). Total RNA was extracted from each sample according to the manufacturer's protocol and re-suspended in 20 µl RNase-free water. The concentration of RNA was determined from the absorbance at 260 nm.

RT-PCR of V_H-Cε transcripts

5 µg RNA was included in a 40 µl cDNA reaction with M-MLV Reverse Transcriptase (Gibco BRL) using an oligo dT primer (Gibco BRL). 5 µl of cDNA was added to a 50 µl PCR reaction, which included each V_H leader region class-specific primer at 0.5 µM (V_H1L-V_H6L) and the Cε1 specific primer (Cε1) at 0.5 µM, dNTP's at 0.25 mM and contained 1.26 U *Pfu* DNA polymerase (Promega). The

reaction was initially denatured at 95°C for 2 min, then subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min, and extension at 72°C for 2 min before a final extension at 72°C for 10 min. 5 μl of the first PCR reaction was then transferred into a second nested PCR, which differed only in that it included V_H class-specific primers homologous to FWR1 (V_H1F-V_H6F) and an internal Cε1 primer (Cε2). This reaction was incubated with an initial denaturation at 95°C for 2 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 2 min, extension at 72°C for 2 min, then a final extension at 72°C for 10 min.

The PCR for amplification of the separate V_H classes of the V_H-Cε transcripts in the PBMC of the non-atopic subject GJ29 employed the same conditions as above, using the appropriate V_H primer in each reaction.

The primers used were as follows:
V_H1L: 5'-CCATGGACTGGACCTGGA-3';
V_H2L: 5'-CAGATGGACATACTTTGTTCCAC-3';
V_H3L: 5'-CCATGGAGTTTGGGCTGAGC-3';
V_H4L: 5'-CGATGAAACACCTGTGGTTCTT-3';
V_H5L: 5'-ATGGGGTCAACCGCCATCCT-3';
V_H6L: 5'-GATGTCTGTCTCCTTCCTCAT-3';
V_H1F: 5'-CAGGTGCAGCTGGTGCAGTCTG-3';
V_H2F: 5'-GTCTTGTCCCAGGTCAACTTAAGGGAGTCTT-3';
V_H3F: 5'-GAGGTGCAGCTGGTGGAGTCTG-3';
V_H4F: 5'-CAGGTGCAGCTGCAGGAGTCGG-3';
V_H5F: 5'-GAGGTGCAGCTGCTGCAGTCTG-3';
V_H6F: 5'-CTGTCACAGGTACAGCTGCAGCAGTCAG-3';
(Based on V_H region primers published previously (25, 26))

Cε1: 5'-TGTCCCGTTGAGGGAGCCTGT-3';
Cε2: 5'-GGGTCGACAGTCACGGAGGTGGCATT-3',
(Based on Cε primers published previously (27))

RT-PCR of V_H-Cμ, V_H-Cα and V_H-Cγ transcripts
PCR amplification of sequences from IgM^+, IgG^+ or IgA^+ B cells was carried out as separate reactions based on a multiple step PCR published previously (28, 29).

PCR 1
The initial nested PCR was carried out as for the amplification of V_H-Cε except that only the V_H region primer specific to the V_H class of interest was used; e.g. reaction 1 utilised V_H5L in conjunction with each of the individual outer C_H primers (Cμ1, Cα1 or Cγ1). All other conditions remained the same as those specified above for IgE and the reaction denatured at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min, extension at 72°C for 2 min and a final extension of 72°C for 15 min.

PCR 2
Reaction 2 of the nested PCR for amplification of sequences from IgM^+, IgG^+ or IgA^+ cells utilised an inner V_H5 primer, V_H5Fm, with each of the inner C_H primers (Cμ2, Cα2 or Cγ2) to further amplify the V_H5-C_H sequences. All other conditions remained the same except initial denaturation was carried out at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 2 min, extension at 72°C for 2 min, then a final extension at 72°C for 15 min.

The PCR products from each second PCR were subjected to electrophoresis on a 1% agarose gel and the DNA excised. 5 μl of the gel extracted PCR product was carried forward into a third, semi-nested PCR for each isotype.

PCR 3
Reaction 3 was semi-nested, utilizing the gel extracted PCR 2 products with the inner of each set of C_H primers and a CDR3/FWR4 primer specific for each clonal family (e.g. B16V5 specific for the SO16 V_H5 family) to amplify from CDR3 to the 5' of C_H. The conditions for PCR 3 were as detailed above except the initial denaturation 95°C was for 2 min, then 20 cycles of denaturation at 94°C for 1 min, annealing at

53°C for 2 min, extension at 72°C for 2 min, and a final extension at 72°C for 15 min. PCR products were cloned and sequenced as below before proceeding to the following stage.

PCR 4

In order to obtain the V_H region sequences from specific IgA clones, 5 μl of the gel extracted PCR 2 products was used in the semi-nested reaction 4 with conditions as for the previous reactions, except for the use of the VH5Fm primer and SO16A4 (specific for the D-J junction of the family) with initial denaturation at 95°C for 2 min, then 20 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 2 min, extension at 72°C for 2 min, and a final extension at 72°C for 15 min.

PCR 5

An additional experiment was carried out to re-amplify the complete sequence of the V_H5-IgA clone 1 from CDR1 in the V_H region through the D and J_H region to Cα (previously only analyzed by PCR 3 and 4 generating different fragments of the full sequence). The semi-nested PCR 5 included; 5 μl of the gel extracted PCR 2 products with conditions as above, except that the specific CDR1 primer (B16V5C1) was used in conjunction with the inner Cα primer. The reaction was then incubated for an initial denaturation at 95°C for 2 min, then 20 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 2 min, extension at 72°C for 2 min, and a final extension at 72°C for 15 min.

The primers used were as follows:
Cμ1: 5'-GCTCGTATCCGACGGGGAAT-3'
Cμ2: 5'-CGAGGGGGAAAAGGGT-3'
Cα1: 5'-GGGACCACGTTCCCATCT-3'
Cα2: 5'-CTCAGCGGGAAGACC-3'
Cγ1: 5'-CGGTTCGGGGAAGTAGTCCTT-3'
Cγ2: 5'-CAGGGGGAAGACCGAT-3'
(Cμ2 and Cα2 based on that published previously (30, 31))

VH5Fm; 5'-TGCAGCTGCTGCAGTCTG-3'
B16V5; 5'-AGACATAAGAGTGGCTCG-3'
SO16A4; 5'-TGGCCCCAGTAGTCAGC-3'
B16V5C1; 5'-TATAAGTTTGCCACCTATGCC-3'

RT-PCR of AID transcripts

To amplify AID mRNA transcripts by RT-PCR, cDNA was manufactured from nasal biopsy samples taken from seven allergic rhinitis patients as above. 5 μl cDNA was added to each 25 μl PCR reaction, which included the forward primer AID1P at 0.2 μM and the reverse primer AID2P at 0.2 μM, dNTP's at 0.2 mM, $MgCl_2$ at 1 mM and included 1.25 U Platinum *Taq* DNA polymerase (Invitrogen). The reaction was initially denatured at 94°C for 5 min, then subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min before a final extension at 72°C for 5 min. 5 μl of the first PCR reaction was then transferred into a second PCR, which differed only in that it included an inner forward primer AID3P at 0.2 μM and an inner reverse primer AID4P at 0.2 μM. This reaction was incubated with an initial denaturation at 94°C for 5 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min, then a final extension at 72°C for 5 min.

Details of the primers are as follows;
AID1P: 5'-GAGGCAAGAAGACACTCTGG-3'
AID2P: 5'-GTGACATTCCTGGAAGTTGC-3'
(Based on AID primers published previously (17))

AID3P: 5'-TAGACCCTGGCCGCTGCTACC-3'
AID4P: 5'-CAAAAGGATGCGCCGAAGCTGTCTGGAG-3'
(Based on AID primers published previously (32))

Cloning and sequencing of PCR products

All PCR reaction mixes were subjected to electrophoresis on 1% agarose gels except for the Cμ, Cα and Cγ PCR products from reaction 3 and the AID PCR products which were subjected to electrophoresis on 2% or 1.5% agarose gels, respectively. Bands of the appropriate size were gel purified (Qiagen) and cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). *Eco*RI digests confirmed the presence

of the cloned insert and subsequent minipreps were sequenced using M13F or M13R primers (Molecular Biology Unit, KCL, UK and ABC Sequencing Service, IC, UK).

Identification of sequences

Assignment of V_H, D and J_H genes and their somatic mutations was carried out according to their homology with the germline sequences detailed on the VBase database (www.mrc-cpe.cam.ac.uk). The identity of a D gene given a score of less than 50 by VBase was regarded as undefined (where +5 is awarded for a nucleotide match and –4 for a mismatch). Where the alignment of the V_H and D gene or D and J_H were seen to overlap, precedence was given to V_H and J_H so that a truncated D gene sequence was presented. The CDR3/FWR4 region of each sequence was used to determine clonality. Genealogical trees were constructed based on the premise that shared mutations were acquired early, and individual mutations acquired later in the evolution of the clone. Antibody isotype and AID sequence homology was established using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Results

V_H sequence analysis and IgE$^+$ B cell clonality

We have analyzed V_H-Cε sequences from the nasal biopsy samples of six patients (five multiallergics and one grass pollen monoallergic), characterized by skin-prick test, RAST and a medical history of allergic rhinitis for at least two years (Table I). The selected patients exhibited serum IgE levels >200 IU/ml to ensure success in RT-PCR amplification of V_H regions (27, 33). Fifteen sequences were analyzed from the nasal biopsy samples of each patient.

INSERT TABLE I

All samples were PCR amplified using *Pfu* DNA Polymerase, which exhibits a proof-reading ability resulting in a very low experimental error rate compared to other DNA polymerases. The experimental error rate generated in our system of nested RT-PCR amplification was determined to be approximately 1 in 4720 bp, the equivalent of 1 mutation introduced experimentally in approximately every 17 V_H region sequences amplified in this manner. Experimental error rate was determined by analysis of the number of mutations introduced into the Cε DNA amplified by the V_H-Cε PCR (data not shown).

Initial analysis of V_H-Cε sequences generated by RT-PCR demonstrated that we were able to detect all V_H gene families with the exception of V_H2, one of the most infrequently expressed V_H gene families (34). V_H, D and J genes were assigned using the VBase database (www.mrc-cpe.cam.ac.uk), allowing mutations from the germline to be identified.

V_H-Cε sequences containing mutations from the germline that followed the expected trends of SHM were isolated from all nasal biopsy samples (Table II). Multiple copies of identical sequences were isolated from all samples. All sequences contained in-frame rearrangements, implying successful translation of mRNA into IgE protein.

INSERT TABLE II

Detailed analysis of V_H region sequences sharing identical CDR3/FWR4 regions was carried out in each sample, allowing the construction of families of clonally related IgE$^+$ B cells (nucleotide sequences submitted to EMBL, accession numbers AJ491904 – AJ491915). Due to the experimental error rate in our system, two families (each comprising only two B cell clones) were discarded on the basis that the single mutations linking each of the related clones may have been experimental artefacts. When PBMC from each of the patients was analyzed (and three small families, again, each comprising two clones linked by a single nucleotide difference, were discarded) only two distantly related B cell clones were detected (from patient HD17, who exhibited the highest total serum IgE of all the patients) and which differed by nineteen V_H mutations, four D and one J_H mutation while still exhibiting clear homology across the 'signature region' (data not shown).

Clear evidence of clonally related IgE$^+$ B cells, closely linked by multiple differences in their V_H mutations, was however evident in two of the six nasal biopsy samples analyzed (patients SO16 and AP19). Sequences suggesting two families of related IgE$^+$ B cell clones were isolated from SO16. One family of B cell clones expressed a V_H3 gene and comprised three B cell clonal members (of which one was a hypothetical intermediate, not isolated experimentally). The other family of B cell clones isolated from SO16 expressed a V_H5 gene and comprised four B cell clones, of which one was a hypothetical intermediate. The alignment of these related clones (*Fig.* 1) enabled the formation of 'genealogical trees',

demonstrating the clonal relationships between the B cells (*Fig.* 2). In only one instance, did sequence data suggest the presence of an IgE^+ B cell clone in the PBMC that was distantly related to B cell clones detected in the nasal mucosa (one clone from the PBMC of SO16 shared five mutations and the same V_H-D-J_H rearrangement (data not shown) with the family of V_H3 IgE^+ B cell clones depicted in *Fig.* 2A).

Identification of related IgE and IgA B cell clones in the nasal mucosa

Further investigation of patients SO16 and AP19 was undertaken to determine whether sequences from cells related to the families of IgE^+ B cell clones, but which expressed isotypes other than IgE could be detected in the nasal mucosa. A three-stage PCR approach based on that documented previously (28, 29) was utilised to achieve this, using a nested reaction (PCR 1 and 2) to amplify V_H3 or V_H5 sequences from either IgM^+, IgG^+ or IgA^+ B cells in each sample. The third PCR reaction in each case utilised a semi-nested approach using a forward primer specific for the clonal 'signature region' (V_H-D junction) of interest, combined with the inner set of primers for IgM, IgG or IgA.

No sequences suggesting the presence of B cell clones of any other isotype related to the V_H3-Cε family from SO16, or the V_H5-Cε family from AP19, were detected. In contrast, successful amplification of a 'signature region' from an IgA_2 B cell related to the V_H5 family of IgE^+ B cell clones was isolated from the nasal mucosa of patient SO16 (*Fig.* 3A) (nucleotide sequence submitted to EMBL, accession number AJ491916), indicating the presence of at least one IgA_2^+ B cell sharing a common ancestry with the previously isolated family of IgE^+ B cells.

Another, separate, semi-nested PCR (PCR 4) was used to amplify the full V_H region sequence from the related IgA^+ B cell(s) from which the signature region had been amplified in PCR 3. Sequences were detected from five IgA^+ B cell clones that were related to each other and to the IgE^+ family of B cell clones and which exhibited a complex pattern of shared and unique somatic mutations throughout the full length of the V_H region sequence (nucleotide sequences submitted to EMBL, accession numbers AJ536081 - 5). These sequences were aligned and homology with the sequences from the related IgE^+ B cell clones, also isolated from the nasal mucosa of patient SO16, could clearly be seen (*Fig.* 3B).

In order to eliminate the possibility that the IgA clones were PCR artifacts (unlikely as both the shared and diverse mutations were dispersed throughout the V_H region), we successfully re-amplified in PCR 5 the complete V_H-D-J_H-Cα2 sequence 3' of CDR1 from the IgA clone C1 that had previously been partially amplified by PCR 4 (nucleotide sequence submitted to EMBL, accession number AJ536086) (data not shown). Both the signature region and the somatic mutations were identical to that amplified previously, confirming that the sequences presented here were unlikely to be PCR artifacts.

V_H-Cε sequences were not isolated from the nasal mucosa of a non-atopic subject with highly elevated serum IgE

In order to investigate whether random migration of IgE^+ B cells from the circulation to the nasal mucosa occurs in subjects exhibiting high systemic IgE levels (a possible explanation for the presence of related B cell clones seen in the nasal mucosa of allergic rhinitis patients), a nasal biopsy was taken from non-atopic healthy subject GJ29 who exhibited extremely elevated serum IgE (total serum IgE = 1834 IU/ml). No V_H-Cε sequences could be isolated from the nasal biopsy sample (*Fig.* 4A), although the nasal biopsy exhibited a strong GAPDH signal (*Fig.* 4C) and V_H-Cμ, V_H-Cα, and V_H-Cγ PCR products, corresponding to IgM, IgA and IgG^+ B cells respectively, could be amplified from the sample (data not shown). In addition a strong IgE signal was obtained from PCR amplification of IgE V_H regions from the subject's PBMC (*Fig.* 4A). When V_H-Cε transcripts from the subject's PBMC were amplified using primers to amplify the different V_H class in separate reactions, the subject's IgE was shown to be comprised of B cells expressing V_H1, V_H3, V_H4, V_H5 and V_H6 (*Fig.* 4B), thereby demonstrating that the repertoire of IgE^+ B cells in the PBMC was diverse and that a mono / oligoclonal lymphoproliferative disorder did not account for the subject's raised total serum IgE.

Local AID mRNA transcripts in the nasal mucosa of allergic rhinitis patients.

In order to investigate the presence of local AID mRNA transcripts in allergic rhinitis, a nested PCR technique was applied to nasal biopsy samples from seven patients. Two of these samples (CM10 and AP19) had also been used in the V_H-Cε transcript analysis (restricted sample size unfortunately prevented the analysis of the other five nasal biopsies on which V_H-Cε analysis had been carried out). All of the patients in which AID analysis was studied were either biopsied within the grass pollen season or suffered from perennial allergic rhinitis. When the PCR products were subjected to agarose gel electrophoresis,

AID mRNA was shown to be present in the nasal mucosa of five of the seven patients (*Fig.* 5). The PCR products were confirmed by Southern blot analysis using a probe that spanned exon 3 and 4 (data not shown).

Discussion

Allergic rhinitis is increasingly prevalent in the Western world. Studies at the molecular level have enabled the investigation of IgE-mediated immediate hypersensitivity at specific locations. Previous work has demonstrated the occurrence of local IgE protein production in the nasal mucosa of patients suffering from allergic rhinitis (1), but no PCR based investigation has been undertaken until now to determine whether DNA class switch recombination to generate B cells committed to IgE synthesis and concurrent somatic hypermutation also occur locally in the nasal mucosa of allergic rhinitis patients (as has been shown for the asthmatic lung mucosa (13)). These processes are potential therapeutic targets.

In order to investigate whether local CSR and SHM occur locally in the nasal mucosa of allergic rhinitis patients, we have amplified IgE^+ V_H region sequences by RT-PCR from the nasal mucosa of six allergic rhinitis patients. These sequences have provided clear evidence of families of closely related IgE^+ B cell clones in the nasal mucosa of two of the six patients. This was in stark contrast both to the one instance in which two very distantly related IgE^+ B cell clones were isolated from the PBMC of one patient and the one instance in which an IgE^+ clone from the PBMC was found to be distantly related to a family of IgE^+ clones from the nasal mucosa (possibly resulting from the diffusion of related cells from lymphoid tissue prior to SHM and clonal expansion in the nose, or from the nasal mucosa into the circulation).

Each family of related B cell clones originate from a common precursor B cell that proliferated and was subjected to differing extents of SHM. We propose that the occurrence of local SHM and subsequent clonal expansion is a likely explanation for the presence of these families of closely related IgE^+ B cell clones in the mucosa, rather than the migration of all members of each family of related B cell clones from lymphoid tissue to the same location in the nasal mucosa.

Additional evidence supporting the theory of local events in the nasal mucosa was provided by the further investigation of patient SO16. Sequences were identified from five different IgA^+ B cells that exhibited identical CDR3/FWR4 'clonal signature' regions to those from the IgE^+ family of related B cell clones also isolated from the same nasal biopsy sample. These IgA sequences exhibited a range of mutations shared with and also different from the IgE sequences. Again, it is unlikely that these IgA^+ cells were derived from CSR in lymphoid tissue and that they then homed to the same 2.5 mm^3 of nasal mucosa as the related IgE^+ B cells. Detection of related IgE^+ and IgA^+ B cell clones instead implies that it is more likely that the different antibody isotypes were generated by local CSR as has been previously suggested in the lung mucosa of allergic asthmatics (13).

Further studies were carried out in order to investigate the origin of the related B cell clones observed in the nasal mucosa of the allergic rhinitis patients. Analysis of a healthy non-atopic subject with highly elevated IgE indicated that whereas (as expected) a strong RT-PCR product resulted from the amplification of IgE V_H regions from the subject's PBMC, no IgE V_H region sequences could be isolated from the subject's nasal mucosa sample even though a positive GAPDH signal could be amplified and V_H region sequences from other isotypes were detected. This suggests that a high level of serum IgE (from multiple different IgE^+ B cell clones) does not automatically result in the random migration of IgE^+ cells to the nasal mucosa, although it is possible in allergic rhinitis patients that specific cell recruitment takes place.

AID expression has been shown to be associated with both CSR and SHM (17, 18). The presence of AID in the nasal mucosa might therefore be expected if the local microenvironment supported CSR and SHM. We detected AID mRNA transcripts in the nasal mucosa of five out of seven allergic rhinitis patients, demonstrating for the first time the presence of AID mRNA transcripts in local human tissue and strengthening the suggestion of local CSR and SHM in the allergic nasal mucosa. Unfortunately, restricted sample size meant that we were only able to analyse AID mRNA expression from two of the samples used for V_H-Cε analysis, CM10 and AP19. Interestingly, AID expression was clearly detected in CM10 (in which no reliable evidence of related clones was detected), but was not detected from the nasal biopsy AP19 from which a family of related IgE^+ B cell clones was detected. It is therefore possible that the local activity in AP19 had ceased and that AID expression had subsequently been down-regulated, while the related B cell clones remained.

This investigation is the first to demonstrate the presence of local families of closely related B cell clones in the nasal mucosa of allergic rhinitis patients and to provide clear molecular evidence for the occurrence of localized AID mRNA expression and indirectly, evidence of local SHM, CSR and clonal expansion in an area of the nasal mucosa distant from lymphoid tissue. In addition, we suggest that while the presence of related B cell clones in two out of six nasal biopsy samples and of AID in five out of seven samples is of importance in itself, it is likely that the nasal biopsy samples that appeared negative for related B cell clonal families may have done so as a result of sample variation: It is likely that related B cell clones in the nasal mucosa of allergic rhinitis patients are located in clusters, as observed by immunohistochemical staining of $CD19^+$ B cells from nasal biopsy sections. Our preliminary analysis of adjacent halves of a biopsy has suggested that clonal families are highly localized within the mucosa (Coker *et al.*, unpublished observations). Our inability to take more than a single biopsy at any one time from a patient for ethical reasons implies that such clusters may be excluded by the nature of the random sampling of approximately 0.1% of the inferior turbinate; each turbinate is on average 10 g, and each nasal biopsy, on average, 10 mg.

While this is the first study to investigate the local environment of the nasal mucosa in this number of patients, a previous study being restricted to just two lung biopsies from a single asthmatic patient (13), we suggest that it is highly significant that in many respects a consistent pattern of results is emerging in comparison with that observed in the allergic asthmatic lung (13). Our results additionally describe analysis of the V_H region repertoire of the nasal mucosa of a healthy non-atopic patient with high systemic levels of IgE, but no detectable local mRNA encoding IgE, suggesting that IgE^+ B cells do not randomly migrate to the nasal mucosa from the circulation. We therefore conclude that the cells in the nasal mucosa of allergic rhinitis patients are stimulated with allergen, undergo class switch recombination, somatic hypermutation and expand locally in the presence of AID. Our data strengthen the argument that the mucosa acts as a microenvironment in which cells from the immune system direct, process and remove antigen in a localized manner and we suggest that this is fundamental to the pathogenesis of allergic disease.

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Table I: Patient's clinical and experimental data.

PATIENT	STATUS AT BIOPSY [a]	TOTAL SERUM IgE (IU/ml)	SPECIFIC IgE (IU/ml)[b]	ALLERGIES[c]	NUMBER OF B CELL FAMILIES (NUMBER OF MEMBERS ISOLATED FROM EACH CLONAL FAMILY) IN NASAL BIOPSY	NUMBER OF V REGION SEQUENCES ANALYZED
JB7	O / P	236	G=9.25 C=11.1	HDM, G, C, D, CL	-	15
CM10	O / P	382	HDM=1.09 C=3.36 G=101	HDM, G, M, T, B, C, D, H, CL	-	15
HD14	I / P	787	HDM=2.68 G=101	HDM, G	-	15
SO16	I / P	514	G=101	G, T, B, D,	2 (2, 3)	15
HD17	I	2745	G=101	G	-	15
AP19	I / P	414	HDM=1.23 C=32.10 G=60.20	HDM, G, C, D, H, CL	1 (3)	15
GJ29	Non-atopic	1834	AF=0.71	-	-	-

[a] In-season (I), Out-of-season (O), Perennial (P)

[b] Determined by RAST for the allergens; House dust mite (HDM), Grass (G), Cat (C), Dog (D), Tree (T), *Aspergillus fumigatus* (AF)

[c] House dust mite (HDM), grass pollen (G), mugwort (M), three trees (T), silver birch (B), cat (C), dog (D), horse (H), *Aspergillus fumigatus* (AF), *Cladosporium herbarum* (CL), *Alternaria alternata* (A), determined by skin-prick test

Table II: V_H region sequences isolated from IgE^+ B cells in the nasal mucosa of allergic rhinitis patients.

PATIENT	SEQUENCE	GERMLINE V_H GENE WITH GREATEST HOMOLOGY[a]	% MUTATION FROM GERMLINE	NUMBER OF TIMES ISOLATED
JB7	B7C14	1-46	22.7	1
	B7C9	3-23	5.5	1
	B7C17	3-74	4.8	8
	B7C22	4-04	7.3	5
CM10	B10C7	3-30 / 3-30.5	2.9	1
	B10C13	3-30 / 3-30.5	4.4	1
	B10C1	3-66	10.9	1
	B10C2	3-66	10.7	12
HD14	B14C16	1-02	5.9	1
	B14C9	3-15	9.9	1
	B14C4	3-30	11.0	1
	B14C2	3-30	7.0	8
	B14C11	4-59	6.4	1
	B14C19	5-a	4.5	1
	B14C7	5-51	7.4	1
	B14C12	5-51	4.8	1
SO16	B16C12	3-20	3.3	2
	B16C18	3-23	1.8	1
	B16C2#	3-30	4.1	1
	B16C3#	3-30	5.2	2
	B16C10	3-33	5.6	2
	B16C1	3-65	ND	1
	B16C13	4-39	3.7	1
	B16C5	5-51	2.9	1
	B16C8*	5-51	8.5	1
	B16C11*	5-51	7.7	1
	B16C14*	5-51	7.7	2
HD17	B17C13	1-69	4.8	1
	B17C1	3-30	9.6	1
	B17C16	4-ND	12.1	1
	B17C9	4-30.1 / 4-31	8.4	1
	B17C37	4-30.1 / 4-31	8.8	1
	B17C15	4-30.2	7.7	5
	B17C31	4-30.2	8.5	3
	B17C32	4-59	5.6	1
	B17C7	7-04.1	3.7	1
AP19	B19C16	1-18	2.2	1
	B19C6	4-04	5.2	1
	B19C3	4-30.1 / 4-31	2.5	2
	B19C2$	5-51	7.1	8
	B19C4$	5-51	6.4	1
	B19C8	5-51	ND	1
	B19C14$	5-51	7.5	1

[a] - V_H gene locus detailed such that 5-51 denotes a V_H5 gene

ND - Not determined

*, # and $ denote members of each IgE^+ B cell clonal family

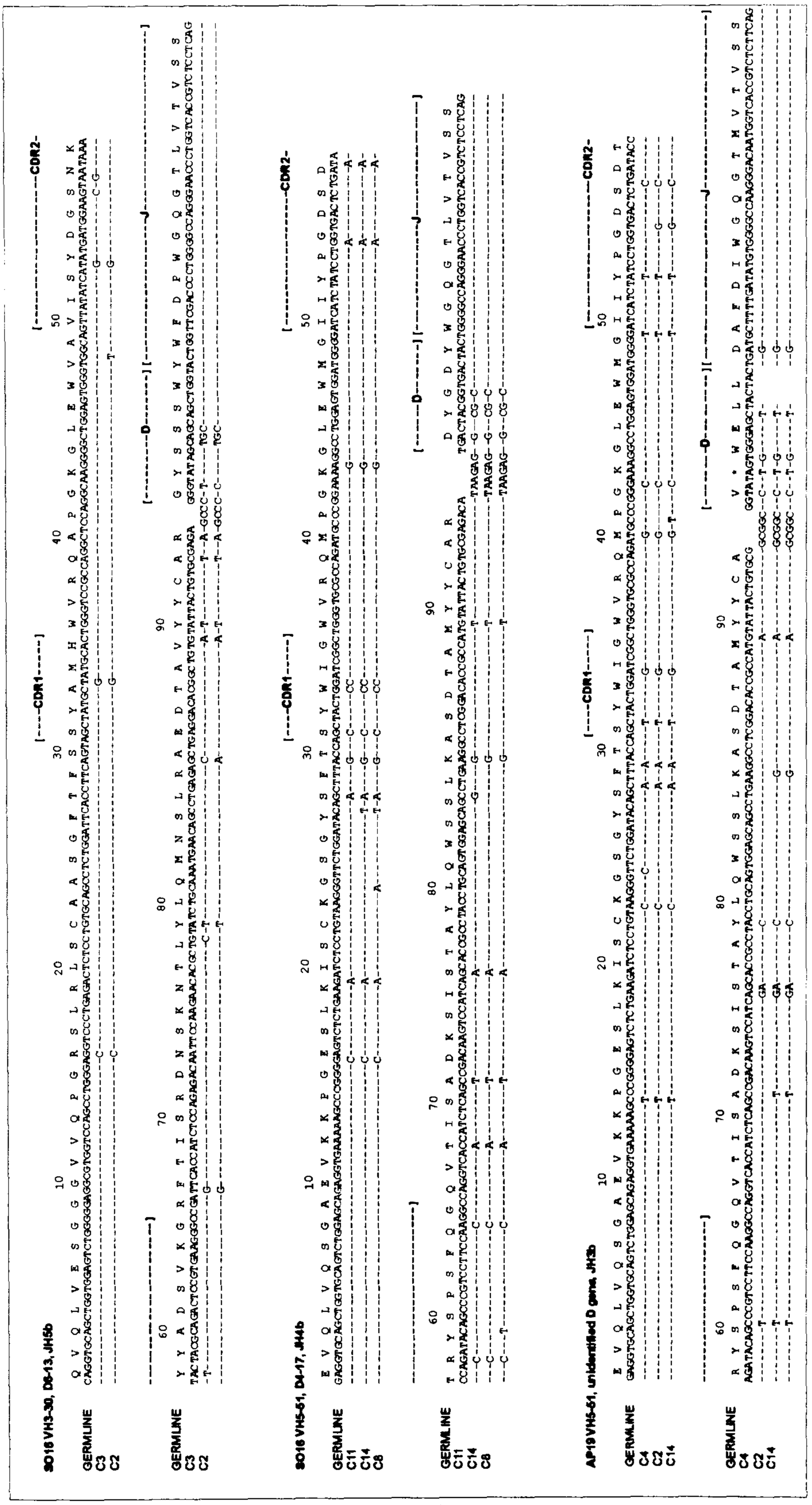

Fig. 1. **Alignment of V_H-D-J sequences amplified by RT-PCR from the nasal mucosa of allergic rhinitis patients SO16 and AP19 indicated the presence of clonally related IgE$^+$ B cells.** Sequences are detailed such that e.g. sequences isolated from AP19 have closest homology with the germline sequence of a V_H gene from the V_H5 family, locus 5-51 (VH5-51), the D gene D1-26 and the J_H gene JH3b. CDR regions are indicated and PCR primer regions underlined. Mutations in the primer regions were not included. (Although the D1-26 germline sequence in reading frame 2 used in AP19 encodes a stop codon, denoted with an asterisk, the non-templated insertion between the V_H and D gene are such that rearranged sequences demonstrate an open reading frame.) CDR regions are indicated and PCR primer regions underlined. Mutations in the primer regions were not included.

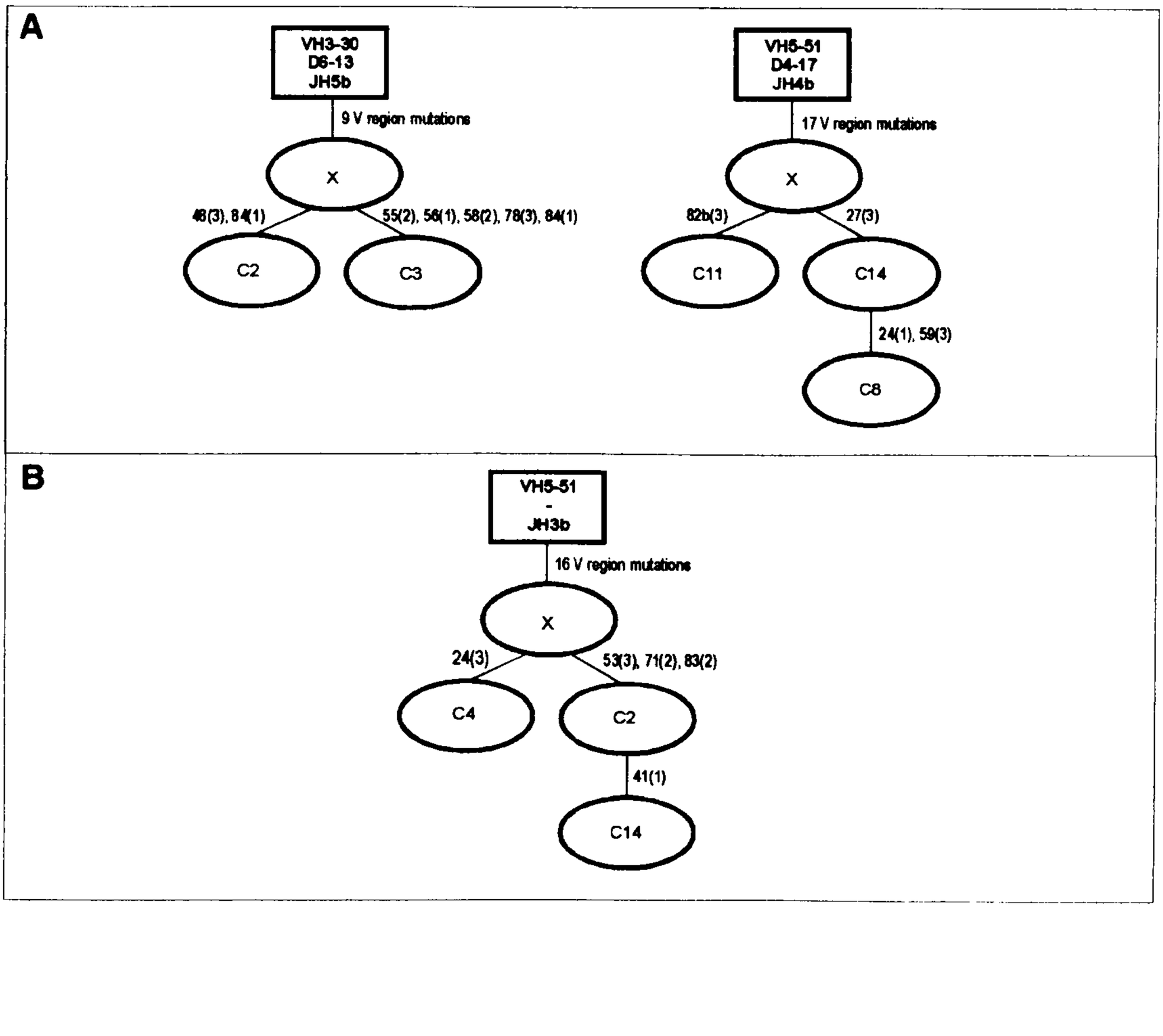

Fig. 2. **Sequences isolated from related IgE$^+$ B cell clones amplified from the nasal mucosa enabled the construction of genealogical trees for allergic rhinitis patients A) SO16 and B) AP19**. The closest germline gene homology is depicted at the top of each tree in a box. Sequences isolated from clones are depicted in an oval. Hypothetical intermediates are denoted with X. Mutations are detailed such that 27(3) represents a single nucleotide substitution occurring at codon 27, nucleotide 3. Each family is detailed such that sequences isolated from AP19 have closest homology with the germline sequence of a V_H gene from the V_H5 family, locus 5-51 (VH5-51), the D gene D1-26 and the J_H gene JH3b.

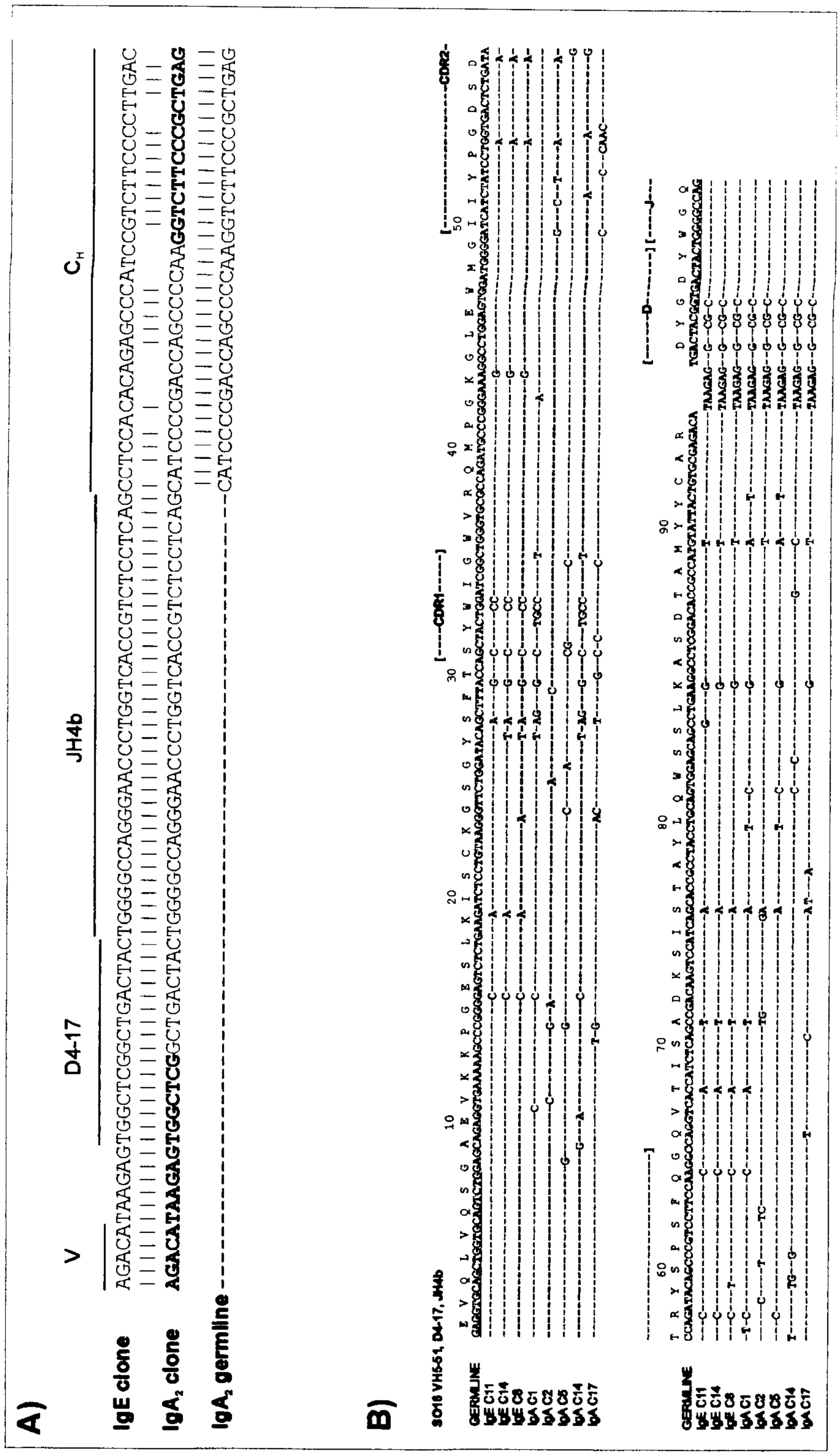

Fig. 3. **Alignment of the signature region and V_H-D-J_H sequence RT-PCR amplified from related IgE$^+$ and IgA$^+$ B cell clones from the nasal mucosa of allergic rhinitis patient SO16.** The signature region sequence (which was joined to the 5' of the IgA_2 C_H region) generated by PCR 3 and also the corresponding V_H-D-J_H sequences from the related IgA clones (amplified by PCR 4) are both detailed, as the join to IgA was only explicit in the signature region sequence. **A)** An identical signature region sequence was amplified by PCR 3 from both IgE and IgA_2 B cell clones. Sequences had greatest homology with the germline V_H5 gene V5-51, D4-17 and JH4b (positions indicated). Primers used in PCR 3 are marked in bold and regions of homology between the sequences are denoted with vertical lines. **B)** The V_H region sequences of the related IgA clones amplified by PCR 4 were aligned with the related IgE clones and exhibited both shared and unique V_H region mutations. The sequences had greatest homology with the germline V_H5 V_H gene V5-51 and D4-17 (indicated). The J_H gene JH4b was only partially amplified and was completely encompassed by the primer region. Primer regions are underlined. Mutations in the primer regions were not included.

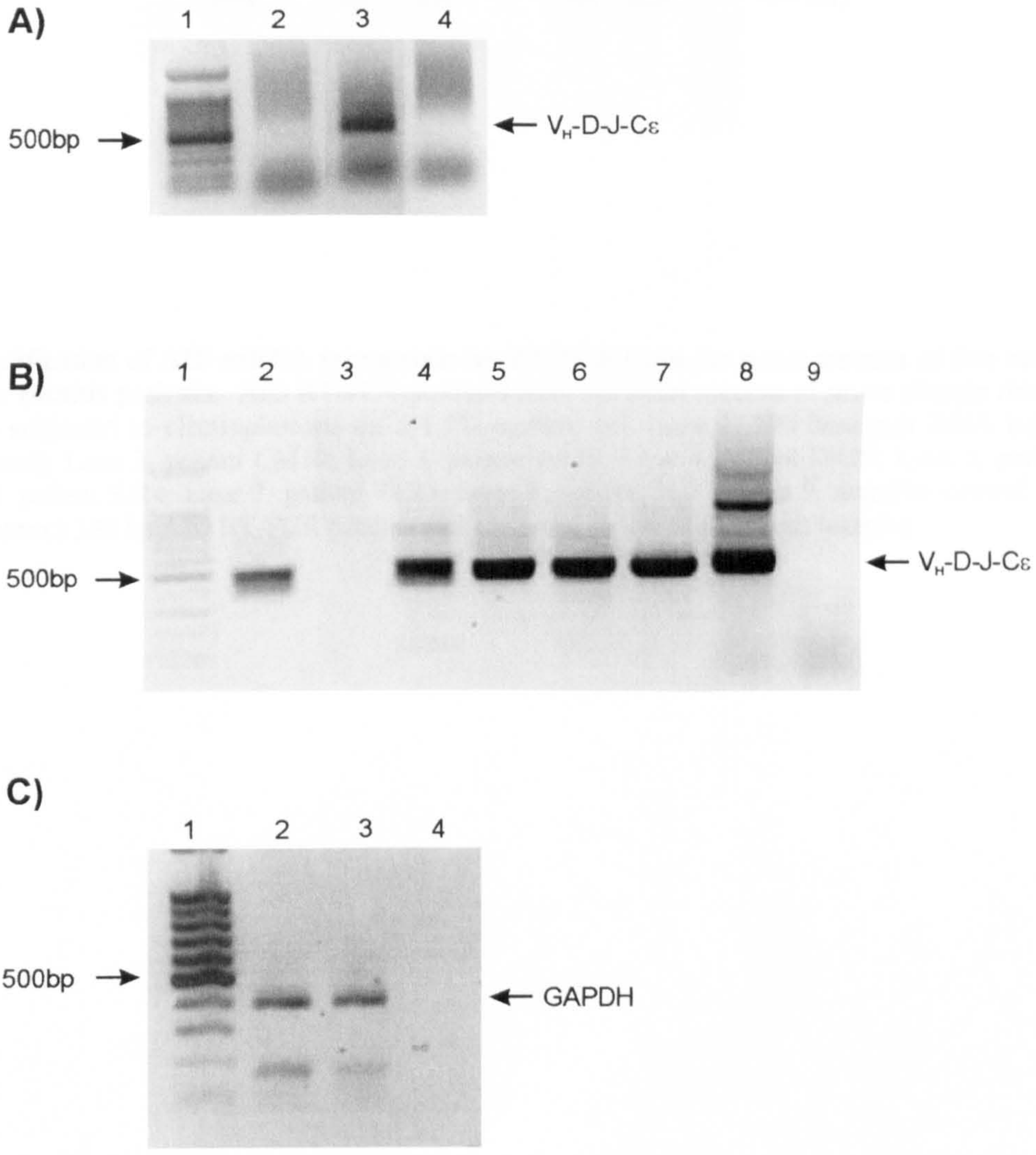

Fig. 4. **V_H-D-J-Cε regions were RT-PCR amplified from the PBMC but not the nasal mucosa of a non-atopic subject with highly elevated levels of serum IgE.** A) V_H-D-J-Cε RT-PCR products were subjected to electrophoresis on a 1% agarose gel; Lane 1, 100 base pair DNA ladder (500bp indicated); Lane 2, non-atopic subject GJ29 nasal mucosa; Lane 3, non-atopic subject GJ29 PBMC; Lane 4, negative control (no DNA). B) V_H-D-J-Cε RT-PCR products from the individual V_H gene classes (V_H1 – V_H6) from the PBMC of non-atopic subject GJ29 were subjected to electrophoresis on a 1% agarose gel; Lane 1, 100 base pair DNA ladder (500bp indicated); Lane 2, V_H1 PCR products; Lane 3, V_H2 PCR products; Lane 4, V_H3 PCR products; Lane 5, V_H4 PCR products; Lane 6, V_H5 PCR products, Lane 7, V_H6 PCR products; Lane 8, all classes of V_H PCR products, Lane 9, negative control (no DNA). C) GAPDH RT-PCR products were subjected to electrophoresis on a 1% agarose gel; Lane 1, 100 base pair DNA ladder (500bp indicated); Lane 2, non-atopic subject GJ29 nasal mucosa; Lane 3, non-atopic subject GJ29 PBMC; Lane 4, negative control (no DNA).

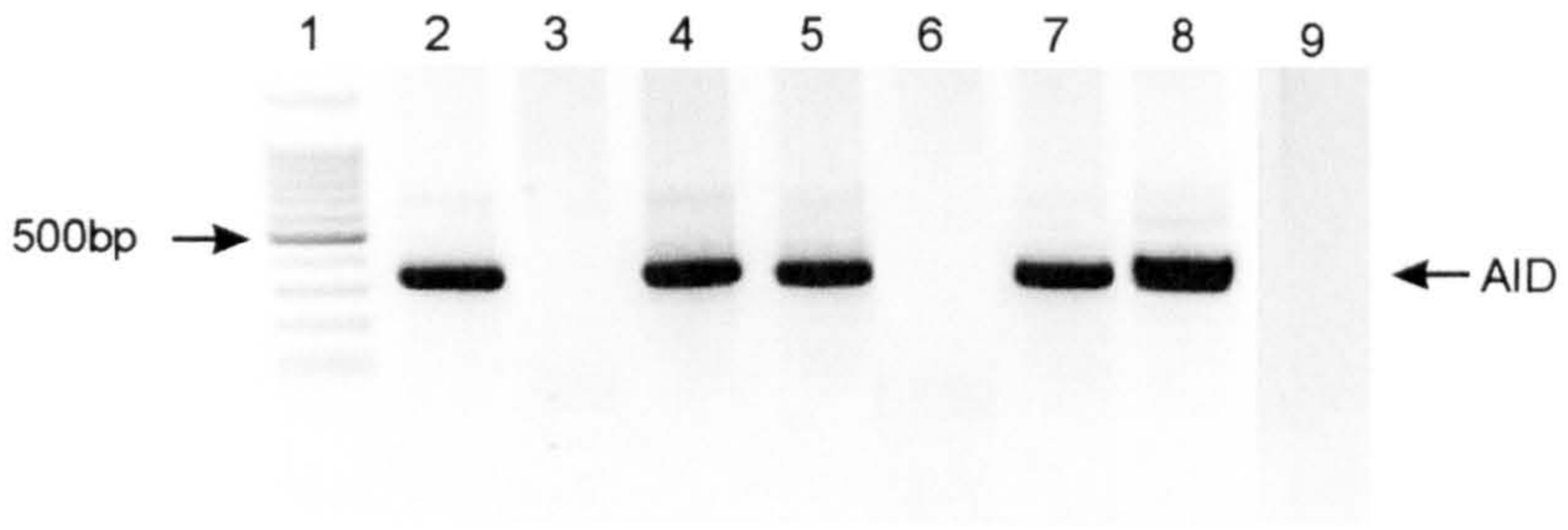

***Fig.* 5. Amplification of AID mRNA transcripts by RT-PCR from the nasal mucosa of five out of seven allergic rhinitis patients.** AID RT-PCR products from the nasal mucosa of seven allergic rhinitis patients were subjected to electrophoresis on a 1.5% agarose gel; Lane 1, 100 base pair DNA ladder (500bp indicated); Lane 2, patient CM10; Lane 3, patient AP19; Lane 4, patient DR20; Lane 5, patient JH23; Lane 6, patient SJ24; Lane 7, patient TL25; Lane 8, patient ZC27; Lane 9, negative control (no DNA). The correct 335 bp AID RT-PCR product was observed in five of the seven samples.